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EFFECTS OF IONIZING RADIATIONS ON A TRANSPLANTED LYMPHOSARCOMA [1] [2]

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INTRODUCTION

A number of investigations have been published in recent years on the effects of chemotherapeutic and physical agents on leukemia in man and experimental animals. This literature has been reviewed by Karnofsky (*1*). Flory, Furth, Saxton, and Reiner (*2*) investigated the effect of chemotherapeutic agents on transplanted mouse leukemia and studied also the effects of irradiation on this disease. They summarize their findings on irradiation of a transplanted mouse lymphoid leukemia as follows: "irradiation . . . with x-rays or gamma rays prolonged life but did not prevent the fatal termination of the disease."

The following experiments deal with the effects of irradiation on a transplanted mouse lymphosarcoma under various experimental conditions: (1) the effect on this tumor of whole-body roentgen irradiation is compared with the effect of the alpha particles from radon and its decay products administered intravenously in comparable energy doses as determined in previous experiments; (2) in addition, the role of the systemic response to irradiation in regression of this tumor is examined.

PROCEDURE

The 45th to 68th generations of a transplanted lymphosarcoma designated L1, which originated in the thymus of a roentgen irradiated strain A male, were used in these studies. The tumor grew well locally at the site of the subcutaneous or intramuscular transplant and never regressed spontaneously. It was palpable about 7 days after transplantation and resulted in death of all mice in 30 to 40 days. Heston line and Bittner line strain A male mice [3] were injected with the tumor at an age of 3 to 4 months and were treated 10 days after transplant.

[1] Received for publication, March 3, 1950.
[2] Presented at a meeting of the American Association for Cancer Research, Atlantic City, April 1950.
[3] Dr. Walter Heston's subline maintained at the National Cancer Institute and Dr. John Bittner's subline maintained at the Medical School of the University of Minnesota.

When work was begun the first aim was to compare the effect on the tumor of whole-body roentgen irradiation with the effect of α-irradiation from intravenously administered radon and its decay products.

An aqueous solution of radon containing 0.1 percent sodium citrate in current equilibrium with its decay products, RaA, RaB, RaC and RaC′, was injected intravenously into the mice to be treated. Amounts given were 0.004, 0.008, 0.012, and 0.016 mc. per gm. yielding energy doses of 0.96×10^4, 1.91×10^4, 2.87×10^4 and 3.8×10^4 ergs per gm., respectively (*3*), or 103, 206, 309, and 412 rep, respectively.

The whole-body roentgen radiation exposures were made over a 2-hour period, and were graded by addition of copper and aluminum filters in order to simulate the dose rate of the radon-treated animals where the RaA, RaB, RaC, and RaC′ emanate for several hours. Hence in irradiation with roentgen radiation 16 percent of the dose was given in the first 10 minutes, 50 percent in the next 50 minutes and the remaining 34 percent in the last 60 minutes. The mice were placed 206 cm. from the anode for these exposures. The tube voltage was 186 kvp. and the tube current 20 ma.

In addition, in some experiments to be described later, short exposures of a few minutes' duration with roentgen radiation were given.

Preliminary Experiment

A preliminary experiment was done in which two groups of mice with subcutaneously transplanted tumors were treated, one with 0.015 mc. per gm. of radon, and the other with 470r roentgen radiation; in addition two groups of normal mice were treated in the same way. White, red, and differential blood-cell counts were taken on these mice as well as on a nontreated group of tumor-bearing mice and a nontreated group of normal mice.

Experiment 1

In the first series, 4 groups of 20 mice carrying the transplant were each given 400r, 300r, 200r and 100r roentgen radiation in the manner described and correspondingly 4 groups of 10 male mice each with tumors were given intravenously 0.016 mc. per gm., 0.012 mc. per gm., 0.008 mc. per gm., or 0.004 mc. per gm. of radon in a 4-hour-old solution. Blood-cell counts were taken on all animals, and several of the experiments were repeated.

Experiment 2

The effect of tumor size at time of treatment on tumor regression was investigated by treating 3 groups of 10 animals each with 300r roentgen radiation. The first was treated on the 10th day after transplant, the second on the 14th day, and the third on the 15th day after transplant. Another experiment was done following the same schedule in which the animals were injected with 0.012 mc. per gm. of radon instead.

Experiment 3

As results indicated that two successive treatments might be additive, two groups of tumor-bearing mice were given 300r roentgen radiation or 0.012 mc. per gm. radon on the 10th and 16th day after transplant. Two more groups were treated similarly on the 10th and 14th day.

Experiment 4

With the completion of the above studies, a second interest, that of the direct and indirect effect of the radiation on the tumor, arose. In one group the tumor and skin flap were shielded with 5 mm. lead, while in another group the mice were put into 5 mm. lead shields and only the tumor and skin flap left exposed. Both groups were irradiated with 400r roentgen radiation. In addition, a third group was irradiated with 400r roentgen radiation with the tumor shielded for all but the last 30 minutes; thus the tumor received only 100r. The dose under the shield in all the experiments was about 1 percent of the total.

Experiment 5

Because Heston strain A mice were not available, the remaining studies were done using Bittner strain A males. A further inquiry into the direct and indirect effects of radiation on tumor growth consisted of: (1) the local roentgen irradiation with 1300r of the lymphomas of a group of lead shielded mice; (2) the local roentgen irradiation of the lymphomas of a second group with 1200r plus whole-body irradiation with 100r; and (3) the local irradiation of the tumor with 1000r plus whole-body irradiation with 300r. Ten animals were used in each experiment. In irradiating the tumor with these high doses the animals were placed in such a way that the tumor was 14.5 cm. from the tube anode and 0.25 mm. copper plus 0.55 mm. aluminum filter were used. During the whole-body irradiation the mice were at a 50 cm. distance, and the roentgen radiation was filtered with 0.12 mm. copper and 0.55 mm. aluminum. The tumor was exposed at the rate of 653r per minute, while the whole-body radiation was given at the rate of 77r per min.

Experiment 6

The possibility that the irradiated body might react to tumor debris in such a way as to enhance tumor regression was investigated. Using mice bearing two subcutaneous tumors, one of the tumors was shielded during a 2-hour exposure to 400r roentgen radiation. In this treatment, if the irradiated body reacting to tumor debris sets up a counter mechanism involving tumor destruction the shielded tumor should respond also.

Experiment 7

Finally, to discover what portion of the body must receive radiation in order that the tumor regresses after irradiation, the tumor was transplanted subcutaneously into either the thoracic, the lumbar, or the sacral region in three groups of mice, and three experiments were per-

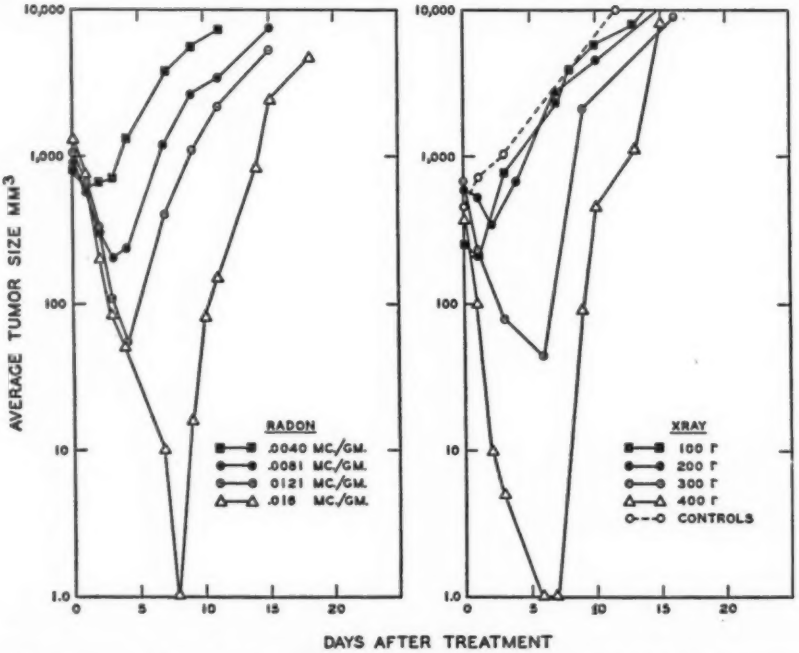
formed in which the mice were irradiated with 400r roentgen radiation given in 2 hours. The mice with the tumor in the sacral region were all shielded with 5 mm. lead from the lower lumbar region up. The mice with the tumor in the lumbar region were completely shielded with 5 mm. lead except for a transverse strip 2 cm. wide in the lumbar area, and the mice with the tumor in the thoracic region were shielded from the lower thoracic region down. White and differential blood-cell counts were made.

Experiment 8

A few studies of the influence of the tumor site on tumor growth following irradiation were made in which the tumor was transplanted intramuscularly into the left hind leg. One group of mice bearing an intramuscular transplant was treated with 0.014 mc. per gm. radon, one with 0.012 mc. per gm. radon, a third with 400r roentgen radiation, and a fourth with 400r roentgen radiation to the body, while the leg bearing the lymphoma was shielded with 5 mm. lead.

RESULTS

Experiment 1 shows that whole-body irradiation with either radon or roentgen radiation produced regression of the lymphosarcoma to a degree dependent on the irradiation dose (text-fig. 1A and B). The differences



Text-figure 1A and B.—Effects of various doses of (A) radon and (B) roentgen radiation on tumor size.

observed were so pronounced that although the individual tumors varied considerably in size, the difference in tumor regression of the varying dose is highly significant for both roentgen radiation and radon. This is shown in table 1 in which the statistical data of the 1st and 3d experiments were tabulated as representatives of all experiments. Results of other and repeated experiments were consistent.

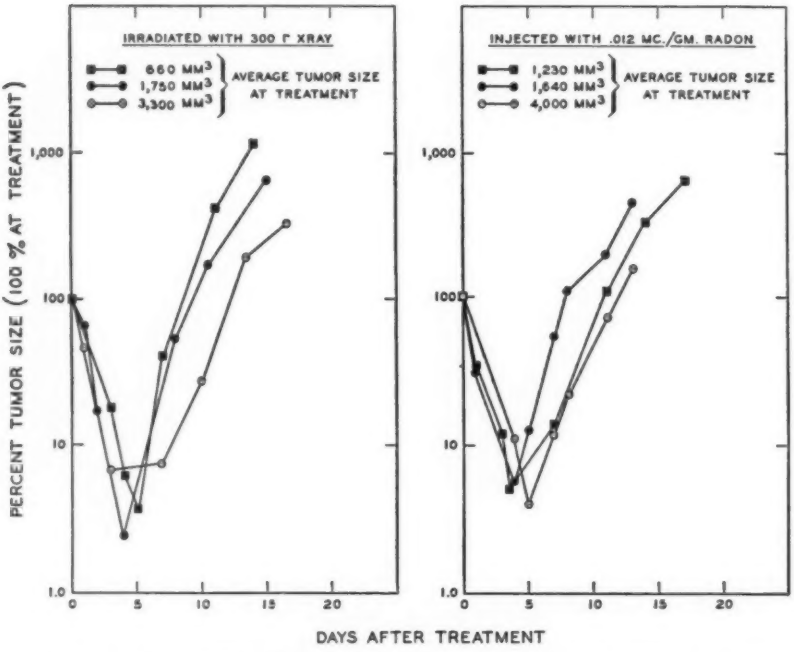
In a few scattered cases in this and the preliminary experiment the tumor regressed completely and did not recur as seen in table 2. Since the LD 50–30 days of radon is about 0.017 mc. per gm. for Heston strain A mice and 0.014 mc. per gm. for Bittner strain A mice, the animals did not tolerate all radon treatments, but the 400r dose was well below the LD 50–30 days of about 580r of roentgen radiation. Whole-body irradiations appeared to be more effective on the intramuscular transplant (experiment 9) than the subcutaneous one. At autopsy, from six months to over a year after treatment, no evidence of the subcutaneous tumor was found in the mice in which the tumor did not recur. Similarly, no evidence of tumor appeared in the leg muscle when the mice of the intramuscularly transplanted lymphoma in which the tumor did not recur were autopsied about 2 months after treatment. Lymphoid cells were found in the muscle but no mitoses were apparent.

Although tumor size on the date of treatment varied considerably, no correlation of the size of the tumor at the time of treatment and the failure of recurrence could be seen. However, in experiment 2 when the mice were treated on the 10th, 14th or 15th day and when the tumors were of considerably different size, the amount of regression, *i. e.*, the tumor size, is proportional to the size of the tumor at treatment as seen in text-figure 2.

Although the calculated doses of radon and corresponding roentgen radiation doses produced equivalent effects on the subcutaneously transplanted tumor, roentgen radiation seemed to be less effective on the intramuscular transplant than was radon (text-fig. 3). Here 0.012 mg. per gm. radon, or 309 rep, caused a greater percentage of the tumors to disappear than did 400r roentgen radiation.

When two radiation treatments were given 6 days apart (experiment 3) the tumor returned in all but one mouse in the roentgen irradiated group. Tumor regrowth commenced before the second treatment. When the two radiation doses were given 4 days apart, however, in five mice in the roentgen irradiated group the tumor did not recur but returned in all mice treated with radon. As only four mice survived two radon treatments, no comparison between the roentgen radiation and the radon effect on the tumor can be made in this experiment.

Whole-body irradiation at doses employed is necessary for tumor regression as shown in table 3. Irradiation of the tumor alone with 400r (experiment 4) produces a tumor regression perhaps slightly greater than that produced by 100r whole-body irradiation, while 400r to the body only produced no regression (text-fig. 4). Irradiation of the body with 400r while the intramuscular tumor was shielded likewise produced no tumor



TEXT-FIGURE 2.—Effect of tumor size at time of treatment on tumor regression.

regression. Moreover, 100r to the tumor while the body received 400r (text-fig. 4) produced no more effect than 100r whole-body irradiation.

In addition, the importance of the direct effect of radiation on tumor growth was emphasized in experiment 5 in which: (1) the tumor alone was irradiated with 1,300r; (2) the tumor alone was irradiated with 1,200r and the animals received 100r whole-body irradiation, or (3) the tumor received 1,000r and the body 300r. Text-figure 5 shows that 1,300r to the tumor alone is no more effective than 200r whole-body irradiation and that 1,000r to the tumor alone plus 300r whole-body irradiation produces the same regression as 1,200r to the tumor plus 100r to the whole body (text-fig. 5), and neither is more damaging to the tumor than 400r whole-body irradiation as seen when these curves are compared with the 400r curve of text-figure 1B.

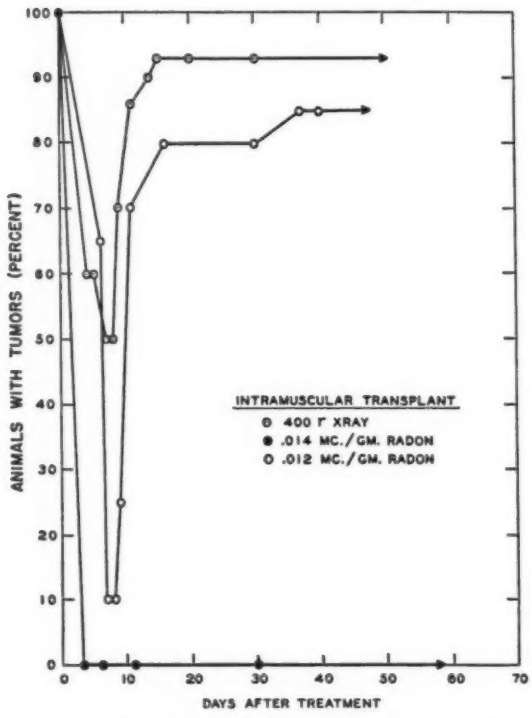
In experiment 6 in which only one of two tumors per mouse was irradiated, the shielded tumor continued to grow at a normal rate, while the unshielded tumor regressed as in experiment 1. Tumor debris, therefore, did not produce a body response which enhanced tumor destruction.

In experiment 7 irradiation of the tumor and the sacral region of the mouse with 400r roentgen radiation produced a tumor regression equal to a 100r whole-body treatment, while treating the tumor in the thoracic or lumbar region with 400r roentgen radiation produced a tumor regression equivalent to that produced by 300r roentgen radiation to the whole body.

TABLE 1.—*Statistical data on experiments 1 and 2*

Experiment	Treatment	Number of animals	Average tumor size at treatment	Percent tumors which become unpalpable	$\frac{\text{Tumor size at treatment}}{\text{Tumor size at minimum}} \times 100$	Level of significance in percent
1	100r x-ray	20	270±170*	0	80±20*	>99
	200r x-ray	20	650±360	0	36±31	>99
	300r x-ray	20	730±470	5	8.9±10.0	>99
	400r x-ray	20	350±210	90	.5± 1.1	
	0.004 mc Rn	10	830±330	0	67±32	
	0.008 mc Rn	10	900±200	0	14± 7.8	>99
	0.012 mc Rn	10	1,630±440	0	5.6± 3.3	99
	0.016 mc Rn	10	1,210±570	80	.3± .6	>99
2	300r x-ray	10	600±270	40	4.7± 5.9	60
	300r x-ray	10	1,720±160	20	3.1± 3.9	30
	300r x-ray	10	3,280±300	0	3.6± 1.9	----------

*S, Standard deviation calculated as $\sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$



TEXT-FIGURE 3.—Effect of irradiation on intramuscular tumor transplants.

TABLE 2.—*Effects of whole-body irradiation with roentgen radiation or intravenously administered radon on a transplanted mouse lymphoma*

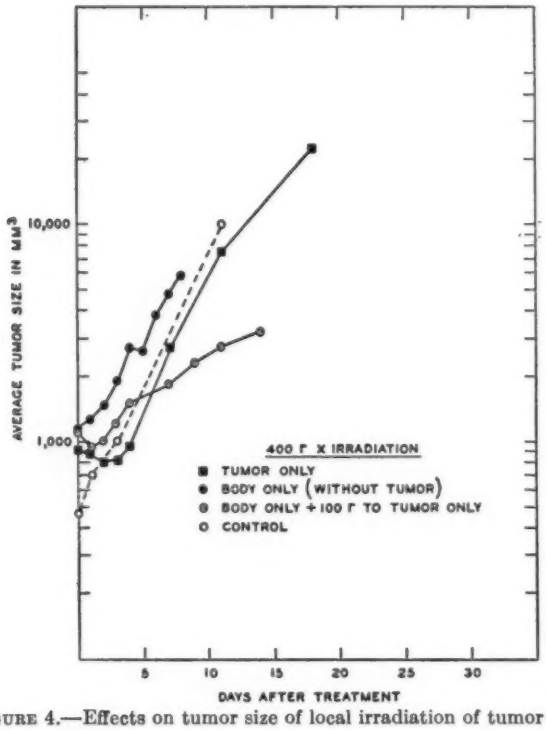
Experiment	Treatment	Number in experiment	Number surviving (15 days after treatment)	Number animals without recurrence (40 days after treatment)
Preliminary	0.015 mc/gm Rn	36	24	4
	470r roentgen rad	20	20	0
1	100r roentgen rad	20	19	0
	200r roentgen rad	69	69	0
	300r roentgen rad	30	30	0
	400r roentgen rad	20	19	3
	0.004 mc/gm Rn	10	10	0
	0.008 mc/gm Rn	10	10	0
	0.012 mc/gm Rn	10	10	1
	0.016 mc/gm Rn	29	7	0
2	300r roentgen rad	30	30	0
	0.012 mc/gm Rn	30	30	0
3	300r+300r roentgen rad 6 days apart.	20	18	1
	0.012 mc/gm+0.012 mc/gm 6 days apart.	13	11	0
	300r+300r roentgen rad 4 days apart.	20	16	5
	0.012 mc/gm+0.012 mc/gm Rn 4 days apart.	16	4	0
4	400r roentgen rad.*	30	29	2
	.014 mc/gm Rn*	10	6	6
	.012 mc/gm Rn*	20	20	3

*Intramuscular transplant.

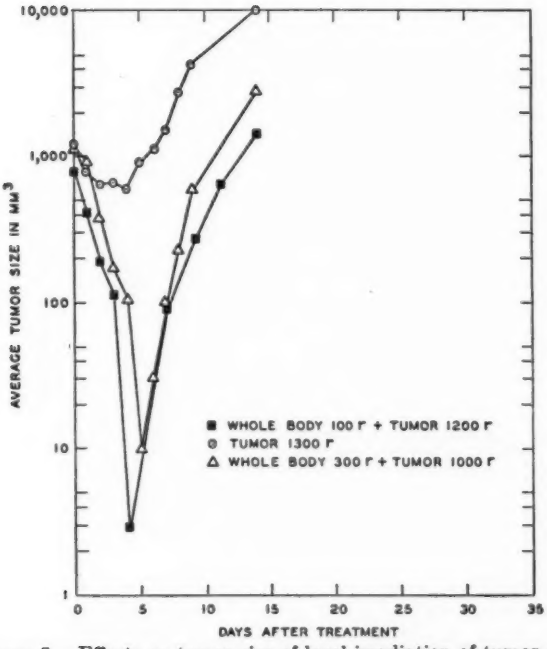
TABLE 3.—*Studies on whole body* vs. *local roentgen irradiation of a transplanted lymphosarcoma*

Experiment	Treatment	Number in experiment	Tumor size in percent when tumor a minimum	Number animals without recurrence (40 days after treatment)
5	400r to body alone	20	100	0
	400r to tumor alone	20	88	0
	400r to body alone 100r to tumor	10	82	0
6	100r to whole body 1,200r to tumor	10	.33	0
	300r to whole body 1,000r to tumor	10	.9	0
	1,300r to tumor alone	10	55.0	0
7	400r to upper body including tumor	10	23	0
	400r to middle body including tumor	8	20	0
	400r to lower body including tumor	8	100	0
8	400r to body and one tumor while second tumor shielded.	10	*100 **0.4	0
9	400r to body without tumor (intramuscular transplant).	10	100	0

*Shielded.
**Not shielded.



TEXT-FIGURE 4.—Effects on tumor size of local irradiation of tumor or body.

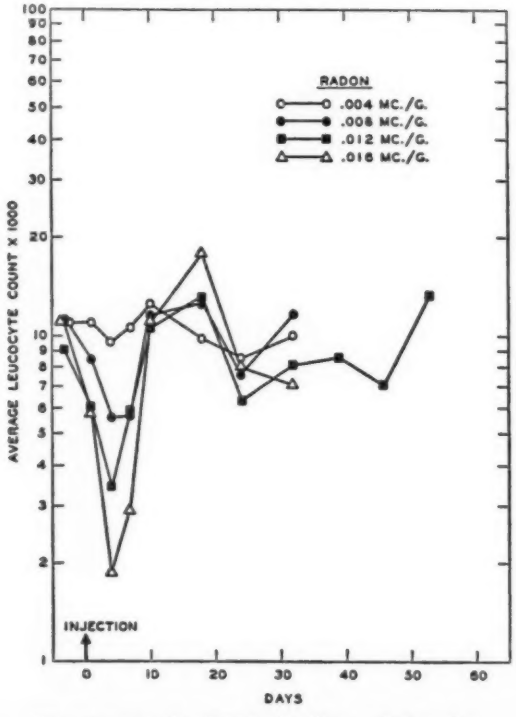


TEXT-FIGURE 5.—Effects on tumor size of local irradiation of tumor or body.

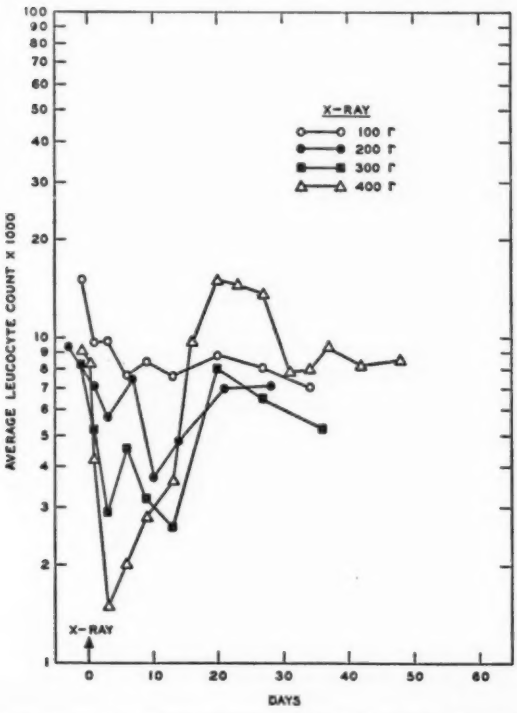
The white blood cell count as well as tumor size is lowered by both roentgen radiation and radon treatment to a degree dependent on the dose (experiment 1) as text-figures 6 and 7 show. Again comparable radon and roentgen radiation doses produce comparable degrees of leukopenia. The recovery of the white blood cell count, however, is quicker after radon treatment than after roentgen radiation treatment. The day following treatment the lymphocyte count dropped, and on the 3d day the heterophil count also dropped. The lymphocytes started to recover on the 7th day as did the heterophils. The presence of the tumor had no effect on the blood counts. The white blood cell counts and the differential counts of the treated tumor-bearing animals did not differ from the blood picture of normal irradiated mice.

When the thoracic, lumbar, or sacral region of the mouse was irradiated with 400r, the white blood cell count increased on the day after treatment and dropped on the 3d day after treatment. This rise is not significant in any group, and the fall is significant only in the group where the animals were exposed from the lower thoracic region up.

Consistently throughout the experiments the red blood cell count dropped 4 days after treatment with 300r or 400r roentgen radiation or with 0.012 or 0.016 mc. per gm. radon, recovered on the 7th day and



TEXT-FIGURE 6.—Effect of radon on leukocytes.



TEXT-FIGURE 7.—Effect of roentgen radiation on leukocytes.

dropped again on the 10th day. The average drop, however, was only two or three million cells per cu. mm. and not significant in any group. When two radiation exposures were given 6 days apart the erythrocyte count fell 4.4 million per cu. mm. for radon treated animals and 3.5 million for mice exposed to roentgen radiation. With the doses given 4 days apart the drop was about 5.5 million for both groups.

DISCUSSION

Although both tumor regression and leukopenic response obtained with the subcutaneous transplant indicate the conversion of absorbed energy of alpha radiation to rep is a valid one, results of the intramuscular transplant studies raise doubts concerning the question of distribution of radon and its decay products in the body as well as the comparability of heavy ionizing particles with ionizing radiation. Certainly the absorption of alpha radiation will not be homogeneous throughout the body. When the tumor is in the muscle, which is well vascularized, the radon is more effective in destroying the tumor than roentgen radiation. From these observations it might be said either that the biologic effects of alpha particle ionization are comparable to those of roentgen radiation ionization and the intramuscular tumor receives more than the average dose or that the

biologic effects are not comparable in which case the subcutaneous tumor would receive less than the average dose. On the other hand, the later experiments where the tumor alone received 1,300r showed that concentration of ionizing radiation in the tumor is not effective in tumor destruction. Furthermore, studies of the radioactive content of the tumor following intravenous injection of radon and its decay products show no preferential concentration in the lymphoma at either location.

At least one other possibility remains: the apparent difference in effect of the two types of radiation on the intramuscular lymphoma may lie in the difference in action of the two types of ionizing radiations. The densely ionizing particles which have a range of 40μ to 70μ in tissue may produce a change in the capillaries of the tumor which is not produced by roentgen radiation, and since the blood supply to the muscle is better than to the dermis and hypodermis, it is possible that the capillaries around the intramuscular tumor are more destroyed by alpha bombardment. To eliminate the possibility that concentration of radioactivity in the muscle causes the difference in response, high doses of roentgen radiation to the leg bearing a transplant might be given along with whole-body radiation. But as the tumor at this site cannot be measured satisfactorily a difference in tumor regression from that following 400r to the whole body probably would not be seen.

More interesting is the role which systemic response to irradiation plays in the regression of this lymphosarcoma. It is evident that two separate mechanisms following irradiation are essential in order to produce regression unless extremely high local doses (above 1,300r) are employed. First of all some reaction of the body is necessary, and secondly the tumor itself must be hit sufficiently. A systemic dose of 100r, which causes only slight leukopenia, is sufficient to produce the action necessary for tumor regression when the tumor is irradiated with 1,200r. On the other hand, when the tumor receives only 100r while the rest of the body receives 400r little damage to the tumor results. The irradiation of no single organ or tissue can account for this systemic effect as was shown when various portions of the mouse were irradiated.

Much has been said about the influence of pituitary-adrenal response on lymphoid tissue. Both whole-body roentgen irradiation and adrenal cortical extract cause involution of lymphoid tissue. Whole-body roentgen irradiation also causes an initial and terminal reduction in adrenal cholesterol (*4*). Dougherty and White (*5*) attempted to show that low dose roentgen in radiation-induced leukopenia was a result of adrenal response to roentgen irradiation when they obtained no leukopenia in adrenalectomized mice following a minimal leukopenic dose of 10r. On the other hand Marder, Kaplan, and Lorenz (*6*) failed to find an effect of adrenalectomy on lymphopenic response in C57 black mice given 30r whole-body irradiation, and Patt *et al.* (*7*) have found a similar leukopenic

response in adrenalectomized and intact rats following 25r whole-body irradiation.

However, Patt, Swift, Tyree, and Straube (*7*) have failed to find a change in the adrenal cholesterol following x-ray doses in the lethal range in hypophysectomized rats although involution of lymphoid tissue was not altered.

Both the spleen and the thymus are sensitive to irradiation, and both have a role in lymphocytogenesis. Although the 100r body dose necessary to cause tumor regression probably causes only very slight damage to the thymus and spleen, certain aspects of the problem involving these organs should be discussed. Kaplan [4] has shown that thymectomy reduces the incidence of leukemia in irradiated animals, and Lorenz (*8*) has shown that shielding the thymus during irradiation decreases the leukemia incidence only slightly in comparison to that of animals given whole-body irradiation. The possibility that the growth of this tumor may be retarded by the absence of the thymus should not be overlooked. Since radiation causes involution of the thymus, irradiated mice might be considered temporarily thymectomized. Moreover, the prevention of tumor growth by a damaged spleen should be investigated. Jacobson (*9,10*) showed that protecting a surgically-mobilized spleen reduced pancytopenia as the uninjured spleen takes on hematopoiesis.

Just which endocrine system or systems, if any, are responsible for producing the systemic effect will be difficult to determine, but that no single organ in any one system can be held entirely accountable is clear.

SUMMARY

When the effect of whole-body roentgen irradiation on a transplanted lymphosarcoma was compared with the effect of alpha radiation of radon and its short-lived decay products injected intravenously, little difference was noticed grossly in the regression curves.

Tumor regression, *i. e.*, the amount of tumor destroyed, was found to be dependent on the dose and was proportional to the size of the tumor at treatment.

The destruction of the intramuscular lymphoma required a higher dose of roentgen irradiation than of radon, (based on conversion to rep).

Irradiation of the whole body was essential in causing marked tumor regression, even when the tumor alone was irradiated with more than three times the whole body dose.

The systemic effect on tumor regression cannot be accounted for by any single organ as shown by irradiating various parts of the body.

Acknowledgment

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[4] Personal communication.

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HOST AND EXTRANEOUS FACTORS IN HETEROLOGOUS TUMOR TRANSPLANTATION [1] [2] [3]

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The results obtained with the transplantation of human cancers into the anterior chamber of the guinea pig eye vary among different types of cancers, among cancers of the same type, and among grafts taken from the same cancer.

In Schilling, Snell, and Favata's (*1*) series successful grafting was more frequently accomplished with nonepithelial cancers than with carcinomas. This also is the experience of the authors (*2*).

Schilling *et al.* (*1*), in their first transplant generation observed growth with tissue from 2 of 6 melanosarcomas, 1 of 2 lung carcinomas, and 1 of 7 breast carcinomas. Hovenanian (*4*) observed growth with material from 2 of over 40 prostatic carcinomas. Eichwald (*2*) obtained growth with material from 1 of 2 fibrosarcomas, and 1 of 13 Wilms' tumors.

Of 10 guinea pigs inoculated with tissue from one lung carcinoma by Schilling *et al.* (*1*), only 1 showed growth. Of 18 animals inoculated by Eichwald (*2*) with tissue from 1 fibrosarcoma only 1 showed growth.

It is not apparent why nonepithelial cancers should grow more readily than carcinomas, why one fibrosarcoma should be more suitable than another, or why grafts from the same cancer should not grow equally well. The last of these three problems lends itself more readily to investigation than the first two, since there are fewer variables. Until the factors are known that influence and decide the fate of grafts taken from one cancer, it is premature to investigate why one cancer is transplanted successfully whereas another is not, or why, in general, sarcomas grow more readily than carcinomas.

The purpose of this study is to determine the factors that significantly influence the fate of grafts from the same cancer when transplanted heterologously into the anterior chamber of the guinea pig eye.

Human cancers are not suitable for a study of this kind because their successful transplantation is not achieved with ease. A tumor was selected which could be transplanted homologously and which had passed through a large number of transplant generations. A tumor most suitable for study would be one in which growth in the anterior chamber of a foreign host would occur under standard conditions in about one half the

[1] Received for publication, March 17, 1950.

[2] Supported by a grant obtained from the National Cancer Institute, U. S. Public Health Service.

[3] Reviewed in the Veterans' Administration and published with the approval of the Chief Medical Director. The statements and conclusions published by the authors are the result of their own study and do not necessarily reflect the opinion or policy of the Veterans' Administration.

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transplants. Factors favorable or adverse to growth would thus be easily recognized. The mouse Sarcoma 37 fits this description well, and was chosen for this study after investigation of a number of other mouse tumors.

MATERIALS AND METHODS

The control host animals were male and female guinea pigs of various ages obtained from local dealers. They were fed a Red Star rabbit-pellet diet regularly supplemented with ascorbic acid. The tumor used in these experiments was mouse Sarcoma 37, which was carried in animals of the C57 black, the dba subline 2, the ABC strains,[4] the CFW strain,[5] and Swiss mice from the Dutch Farms, N. J. The method used for inoculating the foreign host was essentially that reported by Greene (*5*) and others. Local anesthesia only (Cocaine hydrochloride, 7.5 percent) was used; the corneas were incised with a keratome. The tumor tissue was removed aseptically, and, for the control animals, grossly viable portions were set aside in a humidified Petri dish. Grafts were cut to fit snugly into the barrel of a #17 spinal needle. Their approximate size was between 5 and 15 cu. mm. The cornea was cut to permit introduction of most of the beveled surface of the needle. Test and control animals were grafted alternately, from the same tumor fragment, with the same tools and by the same operator.

The animals were observed daily, or every other day, and the observations recorded. Maximal increase in graft size was noted between the 6th and the 20th day, and was invariably followed by regression. Invasion of the periocular tissues was not observed and that of the cornea only rarely. The animals were sacrificed at the time when incipient regression of transplants was apparent, evidenced by beginning gray-yellow discoloration of pink tumor tissue. The eyes were fixed in formalin and sectioned midsagittally through the center of the area of apparent tumor growth.

It was obvious at an early date that owing to the large number of factors affecting growth of transplants the significance of each could be determined only by statistical analysis of results obtained from experiments on large groups of animals.

An arbitrary system for grading the growth of transplanted tumors was established because at the time of beginning regression the amount of tumor present varied considerably.

The following code was adopted (figs. 1–5):

Complete filling of the anterior chamber with tumor tissue in at least one microscopic section	4+
Tumor tissue in excess of one low-power field (magnification 1:100) in at least one section, but not filling the anterior chamber	3+
Tumor tissue less than one low-power field, and filling it approximately halfway	2+
Vascularized tumor tissue distinctly less than one-half of one low-power field in size	1+

A scattering of apparently viable tumor cells, without evidence of vascularization was called ½+. Intermediate degrees of growth were classified as 1½, 2½ or 3½.

[4] Obtained from Bar Harbor, Maine.
[5] Obtained from The Carworth Farms, New City, Rockland County, N. Y.

An estimate of the growth of the tumor in each group of animals was obtained by averaging the degree of growth observed in the individual animals. This average is reported as mean growth.

The highest possible mean growth was 4.0, and the lowest 0.0. The mean growth of all control animals was about 1.1; the highest mean growth in our series was 2.29; and the lowest 0.25.

Following conventional procedure, differences were regarded as being significant when the probability of their being due to chance was 0.05 or less ($P \leqq .05$).

The data of tables 2, 4, 7, 8, 9, 10, 11 are taken from all control groups, and those experimental groups which did not differ appreciably from their control groups.

EXPERIMENTS

Factors conceivably influencing the fate of heterologous grafts were grouped into two main categories: those of an extraneous nature and those concerning the recipient host. Extraneous factors pertain to the physical and biological properties of the inoculum (age, size, degree of necrosis, *etc.*) and to the nature and degree of the grafting trauma. Host factors are either systemic and pertain to the sex, age, and strain, and to the endocrine, nutritional, and immunological status of the host, or are local, and concern the immediate relation of the graft to the host tissue at the transplantation site. This is mainly the ability of the latter to fix and vascularize the graft.

An investigation of extraneous factors shows how far technical inadequacies are responsible for unsuccessful transplantation so commonly seen in this field of investigation.

EXTRANEOUS FACTORS

A. Graft Properties

Donor strain. The strain of the donor mouse (see Materials and Methods) was not found to be a significant factor in influencing tumor growth in the heterologous hosts.

"Immunized" donors. A few donor mice, prior to their grafting with Sarcoma 37, had been unsuccessfully inoculated with mouse Neuroblastoma C 1300. No significant difference in growth was found between tumor tissue carried by these mice and that carried by other mice.

Suspension vs. grafts. A group of guinea pigs was injected with a 25–40 percent suspension of tumor cells in sterile Tyrode's solution, using a #27 needle. The cornea was slightly nicked with a knife before introducing the needle to permit the gradual escape of anterior chamber fluid while the suspension was settling out at the bottom of the anterior chamber. This method prevented a rapid escape of chamber contents on removal of the needle. The data in table 1 show that tumor growth could be achieved when a suspension was used, but solid grafts grew significantly better. Control groups of susceptible mice inoculated with the suspension into the anterior chamber invariably developed tumors.

TABLE 1.—*Graft vs. suspension*

	Number of animals	Mean growth	S. E.
Suspension	40	0. 29	±0. 113
Solid grafts	33	. 95	±. 214
		P=0. 007	

Graft size. A group of guinea pigs was inoculated with grafts 1 cu. mm. or less in size while the controls were given the standard size grafts (5–15 cu. mm). No significant difference was found.

Time of grafting. The control guinea pigs of all experiments were divided into groups according to the time that had elapsed between the removal of the tumor from the donor and its implantation in the new host (table 2). There appeared to be a significant difference between the tumor growth in animals grafted during the first 14 minutes and those grafted later, the latter being less transplantable than the former. This initial drop in transplantability was not followed by a continuous decrease during the remainder of the first 2 hours after donor death. Table 2 shows a comparison of the animals inoculated during the first 14 minutes with all those grafted subsequently.

To determine whether or not the high tumor growth in the animals grafted during this early period was due to the presence of a small number of animals with abundant tumor growth, or if it was a persistent feature with animals inoculated that early, the tumor growth was plotted against 1-minute instead of 15-minute intervals. This analysis revealed consistently high values during the first 13 minutes, in contrast to the animals inoculated subsequently, which showed considerable fluctuation between high and low values.

Since these data were compiled from a large number of experiments one should hesitate to accept the difference as significant.

Age of tumor. The age of the individual tumor at the time of sacrifice of the donor did not significantly influence its growth in the heterologous host.

TABLE 2.—*Time of grafting and transplantability*

Time after sacrifice of donor	Number of animals	Mean growth	S. E.
0–14 min	30	1. 57	±0. 206
15–29 min	98	1. 12	±. 123
30–44 min	101	. 90	±. 122
45–59 min	96	1. 33	±. 133
60–74 min	87	. 91	±. 125
75 and more	55	1. 27	±. 179
15 min. and later	437	1. 09	±. 060
		P=0. 028 (0–14 min. *vs.* 15 min. and later)	

TABLE 3.—*Necrosis of tumor and transplantability*

	Number of animals	Mean growth	S. E.
Partially necrotic grafts	35	0. 99	±0. 206
Nonnecrotic grafts	35	1. 01	±. 202
		P=0.92	

Primary tumor and metastasis. Twenty animals were inoculated with tissue from a tumor at its original site, and 20 animals with grafts from an axillary metastasis of the same tumor. There was no significant difference between these 2 groups.

Necrosis of graft. A group of guinea pigs was inoculated with tumor fragments of which one-third to one-half was grossly necrotic, while the controls were grafted with tissue which grossly appeared to be entirely viable. In table 3 it may be seen that there was no significant difference between the two groups.

Bacterial contamination of graft. Representative fragments were taken from the tumor portions used as sources of grafts and cultured in tryptose broth. Compared in table 4 is the growth of the tumors in guinea pigs grafted with tissue which was sterile on culture with others in which bacterial growth was obtained. Also listed are animals grafted with tissue yielding *E. coli* and others where culture of the graft showed *Staphylococcus*. No significant differences were found.

In one experiment, culture of the tumor showed unusually heavy contamination with *E. coli*. In this experiment the tumor grew poorly. This suggests that heavy bacterial contamination of a graft decreased its chance of growth.

Tumor age and bacterial contamination. Fifteen mice of the susceptible ABC strain were inoculated with Sarcoma 37 and sacrificed in groups of 2–4 animals during the 4th and 5th week, the period during which most donors were sacrificed in the experiments. Colony counts of the tumor tissue removed at the time of sacrifice showed marked rise in bacterial contamination with increase in age of the tumor (table 5). Since increase in age of the tumors did not result in decreased growth (see above), these data provide indirect proof that bacterial contamination of tumor tissue, unless excessive, did not affect its transplantability.

TABLE 4.—*Bacterial contamination of graft and transplantability*

Tissue	Number of animals	Mean growth	S. E.
Sterile	144	1. 09	±0. 097
E. coli	27	. 69	±. 240
Staphylococcus	27	. 91	±. 269
Other contaminants	22	1. 34	±. 262
Contaminated (all)	76	. 95	±. 141

TABLE 5.—*Bacterial contamination and tumor age*

	Number of animals	Number bacteria/gm. tumor
20-day	4	3, 000 8, 000 50, 000 1, 000, 000 Mean: 265, 000
23-day	3	60, 000 220, 000 1, 000, 000 Mean: 427, 000
26-day	3	300 1, 000, 000 1, 000, 000 Mean: 667, 000
29-day	3	400 1, 000, 000 21, 000, 000 Mean: 7, 333, 000
32-day	2	2, 600 20, 000, 000 Mean: 10, 000, 000

B. GRAFTING TECHNIQUE

Bacterial contamination at the time of inoculation. The precautionary measures adopted because of their simplicity were: to sterilize the surgical instruments in alcohol, and to lay them out on sterile towels; to paint the donors skin with mercurochrome, removing the excess with alcohol; to dissect portions of the tumor away from the skin surface and the intestinal loops, to select portions grossly free of necrosis; and, prior to incision, to flush the corneal surface repeatedly with Cocaine solution. Additional precautionary measures, such as autoclaving the instruments, shielding of the eye with a sterile sheet provided with a small window, and touching the site of incision with mercurochrome, were soon abandoned as they did not favorably influence the growth of tumor cells.

Contamination after the end of the grafting procedure is not likely as the cut is sealed soon, and any flow will be directed outward rather than into the eye.

Standardized severe trauma. In the control animals it was attempted to keep the length of the incision to a size which would permit introduction of only part of the beveled portion of the trocar. The graft was forced to side-swipe one edge of the incision when pushed into the anterior chamber by the plunger. In one group of animals the incision was made substantially longer to permit introduction of the entire beveled portion. In these animals the tip of the trocar was introduced downward to the level of the lower portion of the iris and rotated 180 degrees to let the bevel face the posterior surface of the cornea before expelling the graft.

In order to determine the relative importance of the introduction and intraocular manipulation of the trocar and that of the length of the incision in their influence upon the growth of the tumors, an additional

TABLE 6.—*Trauma and tumor growth*

Trauma	Number of animals	Mean growth	S. E.
1. Large incision	52	0. 28	±0. 095
2. Insertion of trocar	32	. 25	±. 106
Trauma, both groups	84	. 27	±. 070
Controls	52	1. 23	±. 195
		P <0. 0001 (Trauma, total, *vs.* controls)	

group of animals was provided. In these, the incision was long enough to permit easy introduction of the trocar but the graft was discharged at the site of the incision just as in the control animals. These groups are compared in table 6. In the "traumatic" groups tumor growth was appreciably below that in the control animals. The difference was significant in both "traumatic" groups.

Hemorrhage. Hemorrhage into the anterior chamber or over the corneal surface at the time of inoculation did not affect growth adversely.

Postoperative host reactions. An attempt was made to evaluate the prognostic significance of a number of postoperative local host reactions. The severity of most of these had been determined by an arbitrary grading system.

Prolapse of ocular contents. The data in table 7 indicate that a moderate to severe postoperative prolapse of portions of the vitreous body, the iris, or inflammatory exudate through the operative incision was a bad prognostic sign for tumor growth.

Conjunctival edema. The chances for tumor growth were better when postoperative conjunctival edema remained at a minimum, as shown by the data in table 8.

TABLE 7.—*Prolapse of ocular contents and tumor growth*

	Number of animals	Mean growth	S. E.
Animals with prolapse of ocular contents	69	0. 56	±0. 114
Controls	283	1. 16	±. 071
		P<0.0001	

TABLE 8.—*Conjunctival edema and tumor growth*

	Number of animals	Mean growth	S. E.
Animals with conjunctival edema	31	0. 69	±0. 174
Controls	210	1. 10	±. 084
		P=0. 036	

Haziness of anterior chamber contents. The data in table 9 indicate that moderate to severe postoperative haziness of anterior chamber contents was an unfavorable prognostic sign for tumor growth.

TABLE 9.—*Anterior chamber haziness and tumor growth*

	Number of animals	Mean growth	S. E.
Animals with haziness of anterior chamber contents	96	0. 80	±0. 110
Controls	278	1. 10	±. 072
		P=0.021	

Corneal vascularization. In table 10 a comparison is made of guinea pigs having developed a significant degree of concentric corneal vascularization with others that did not show this reaction, or only a minimum of it. A reaction of this nature was a favorable prognostic sign.

TABLE 10.—*Corneal vascularization and tumor growth*

	Number of animals	Mean growth	S. E.
Animals with concentric corneal vascularization	169	1. 20	±0. 095
Controls	289	. 94	±. 070
		P=0.028	

Focal corneal vascularization. The prognosis for tumor growth was still better when the corneal vascularization was limited to the segment behind which the graft was located. This is shown by the data in table 11.

TABLE 11.—*Focal corneal vascularization and tumor growth*

	Number of animals	Mean growth	S. E.
Animals with focal corneal vascularization	202	1. 25	±0. 085
Controls	98	. 57	±. 100
		P<0.0001	

HOST FACTORS

A. LOCAL FACTORS

Pre-traumatization. Because focal vascularization of the cornea often accompanied tumor growth in the anterior chamber, we studied the effect of traumatization of the future transplant site on tumor growth.

A group of guinea pigs was prepared as follows: 24 hours prior to transplantation a transcorneal stab was made with a pointed Bard-Parker

blade at the lower circumference of the corneo-scleral junction. An attempt was made to irritate the adjacent portion of the iris by gently scraping it with the tip of the knife. At the time of transplantation the graft was placed into this area, which had become slightly congested and cloudy.

There was no significant difference in tumor growth between the pretraumatized animals and their controls.

B. Systemic Factors

Color of guinea pigs. Analysis of the recipient hosts according to the color of their coat failed to show a significant difference in tumor growth among these groups.

Strain of guinea pigs. A group of 17 guinea pigs of the CFC strain [6] was inoculated. There was no significant difference in tumor growth between these animals and their controls.

Sex of guinea pigs. There was no significant difference in tumor growth between male and female guinea pigs.

X-irradiation. A group of guinea pigs was subjected to 125r whole-body irradiation (140 kv., 25 m. a., no filters, 50r per min. output) 3 to 5 hours before inoculation. The data in table 12 do not show a significant difference between these animals and the control group.

Another group received 225r (200 kv., 25 m. a., filters: 0.5 mm. Cu, 1.0 mm. Al). Several animals in this group died from 8 to 12 days following irradiation. In the surviving animals the tumor grew significantly better than in the controls.

Table 12.—*X-irradiation and tumor growth*

Dosage	Number of animals	Mean growth	S. E.
125r	28	0. 97	±0. 230
Controls	26	. 92	±. 223
		P>0. 5	
225r	29	1. 38	±. 226
Controls	38	. 74	±. 179
		P=0. 028	

Nitrogen mustard. A group of guinea pigs was injected intraperitoneally with HN_2. The dosage was 0.75 mg. per kg. It was administered 3 to 6 hours before tumor inoculation. The tumor grew better in the treated animals but the difference was not statistically significant. Another group was injected with a dose of 1.5 mg. per kg. A significant difference in tumor growth was not found (table 13).

Vitamin C deficiency. A group of animals was placed on a Purina-rabbit-chow diet, which is practically Vitamin-C free. The control group was placed on the same diet but supplemented with high doses of ascorbic acid. The animals were grafted when about one-half the de-

[6] Obtained from the Carworth Farms, Inc., New City, Rockland County, N. Y.

TABLE 13.—*Nitrogen mustard and tumor growth*

Dosage	Number of animals	Mean growth	S. E.
0.75 mg./kg	25	1. 46	±0. 268
Controls	26	. 92	±. 223
		P>0. 05	
1.5 mg./kg	23	1. 11	±. 237
Controls	38	. 74	±. 179
		P>0. 05	

ficient animals showed signs of scurvy. The data in table 14 show that the tumor grew better in the control animals. With the number of animals employed the difference was not significant.

TABLE 14.—*Vitamin C deficiency and tumor growth*

	Number of animals	Mean growth	S. E.
Animals on C deficient diet	18	0. 72	±0. 208
Controls	19	1. 18	±. 272
		P=0.193	

Weight of guinea pigs. The body weight of the guinea pigs was not found to be a significant factor in influencing tumor growth in the heterologous hosts.[7]

Newborn animals.—Gheorgiu (*6*) observed in 1918 that subcutaneous transplantation of rat tumors into mice was more successful when newborn animals were chosen as the recipients. A group of newborn animals was inoculated by us within 24 hours after birth. The data in table 15 show a significant difference in tumor growth between these animals and adult controls. This difference appears even more striking if one considers that grafting of a newborn eye is relatively more traumatic than that of an adult eye. (The difference in size between newborn and adult eyes did not significantly influence the grading of the tumor growth as described in Materials and Methods.)

TABLE 15.—*Tumor growth in newborn animals*

	Number of animals	Mean growth	S. E.
Newborn animals	24	2. 29	±0. 246
Adult animals	29	. 50	±. 170
		P<0.0001	

[7] Animals weighing less than 200 gm. were omitted in this analysis because of the difference in tumor growth between newborn and adult animals described in the next paragraph.

TABLE 16.—*Distribution of growth in binocularly inoculated animals*

Right eye	Left eye							
	0	½+	1+	1½+	2+	2½+	3+	3½+
0	28	1	1	---	---	1	---	---
½+	---	1	---	---	---	---	---	---
1 +	3	1	---	---	2	---	---	---
1½+	1	---	---	1	---	---	---	---
2 +	1	---	---	1	1	1	---	---
2½+	2	---	---	---	---	---	1	---
3 +	---	---	---	---	1	---	---	---
3½+	---	---	---	---	---	1	---	---
4 +	---	---	---	---	1	---	---	---

$P < 0.001$

Correlation coefficient: 0.62

Binocular grafting. A group of guinea pigs was inoculated into both eyes in immediate succession. The left eye always preceded the right, but the interval between the two was very brief, averaging about 2 minutes. A group of monocularly grafted animals served as controls.

Had tumor growth been entirely dependent on systemic host factors and on certain graft factors, such as tumor age and donor strain, the binocularly inoculated animals would have shown growth either in both eyes or in none, dependent on the susceptibility of the recipient animal and on the suitability of the tumor. Had tumor growth been entirely dependent on factors pertaining to the grafting technique, it would have occurred in a random distribution: in both eyes, one, or none, according to chance. Had tumor growth been dependent on both groups of factors, it would have occurred either in both eyes, one, or none; but growth in both eyes and none would have been out of proportion more frequent than growth in one eye only. The results are shown in table 16. They bear out the previous observation that various extraneous and host factors influence the fate of the heterologous inoculum.

The correlation between tumor growth in the left and right eyes was 0.62, a highly significant value. This indicates that whatever factors tended to promote growth did so in both eyes at once.

Growth inhibition in binocularly grafted animals. In the last described experiment tumor growth in the eyes of the monocularly inoculated animals occurred more frequently and more abundantly than in the eyes of binocularly grafted animals. This difference was significant (table 17).

TABLE 17.—*Growth inhibition in animals grafted simultaneously in both eyes*

	Number of eyes	Mean growth	S. E.
Monocular	47	1. 31	±0. 19
Binocular	100	. 61	±. 10
		P=0. 0003	

DISCUSSION

The conclusions derived from this study apply only under the conditions of the experiment and to the particular tumor used. They do not necessarily explain the fate of cancer grafts, human or animal. They are examples of factors that may influence heterologously transplanted cancer.

Not all factors which possibly influence the fate of cancer grafts in the anterior chamber of heterologous hosts have been studied by us. In a spot-check fashion a few factors were selected which appeared most likely to influence this type of growth: scurvy was selected of the group of deficiency diseases; only two doses of x-irradiation were used, and administered immediately before inoculation; and only two types of operative trauma were selected out of an unlimited number of possible types.

Considerable differences in tumor growth were found between the various groups of control animals. The values varied from 0.50 to 1.31. This demonstrates that we failed to consider factors that influenced this type of tumor growth. The experiments therefore were not performed under identical conditions. Had all major factors been investigated, the results of each inoculation could now be predicted within reasonable limits; conditions could be devised which would make growth very likely to occur.

It is well to realize that several factors which were found to exert an enhancing or inhibitory influence did not operate under ordinary circumstances of anterior chamber grafting (binocular grafting, severe trauma, high doses of irradiation, and use of newborn hosts).

These studies were conducted with a minimum of variables, using grafts taken from the same tumor though from different transplant generations. It is premature to attempt to explain the fate of grafts from different tumors of the same type, or of grafts taken from different types of tumor as long as we are unable to explain the fate of grafts taken from the same tumor.

SUMMARY

Mouse Sarcoma 37 was transplanted into the anterior chamber of the eyes of 811 guinea pigs in an attempt to determine the factors causing success or failure of this type of transplantation.

Growth is influenced by *graft properties*, by the grafting *technique* and by the *state of susceptibility of the recipient animal.*

The following *graft factors* were not significant: The strain of the donor mouse, the size of the graft, the interval between donor inoculation and sacrifice (age of the tumor), the presence of necrotic portions in the graft, and the presence of bacterial contamination, unless excessive. Tumors grown in mice that previously had been unsuccessfully inoculated with another tumor did not differ significantly from other tumors that had grown in mice not previously inoculated. Metastatic tumor tissue did not differ significantly in its transplantability from a primary tumor.

Solid inoculums grew significantly better than cell suspensions. Tumor tissue grafted during the first 15 minutes after sacrifice of the donor appeared to grow significantly better than that inoculated subsequently. This decline in transplantability was not continuous within the first 2 hours after death of the donor. No inoculations were done later than this period.

The following statements can be made about *grafting technique:* Meticulous aseptic technique did not improve tumor growth beyond that which was obtained by ordinary cleanliness. Major trauma decreased the chances of growth. Postoperative conjunctival edema, haziness of anterior chamber contents, and prolapse of ocular structures through the incision often heralded failure of the transplantation. Operative hemorrhage did not influence growth adversely. Corneal vascularization, diffuse or limited to the portion overlying the graft, was a favorable prognostic sign.

The following statements can be made about *host factors:* The strain, color, and sex of the recipient host were not significant factors. Nitrogen mustard and irradiation in safe doses did not significantly influence tumor growth. Sublethal doses of x-radiation influenced tumor growth favorably. A low vitamin C intake did not influence tumor growth significantly. Newborn guinea pigs were significantly more susceptible than adult animals. The chances of growth in one eye were significantly decreased if the other eye had been inoculated simultaneously.

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PLATE 1

FIGURE 1.—No. 2023. Almost the entire anterior chamber is filled with tumor tissue: 4 plus. × 13.

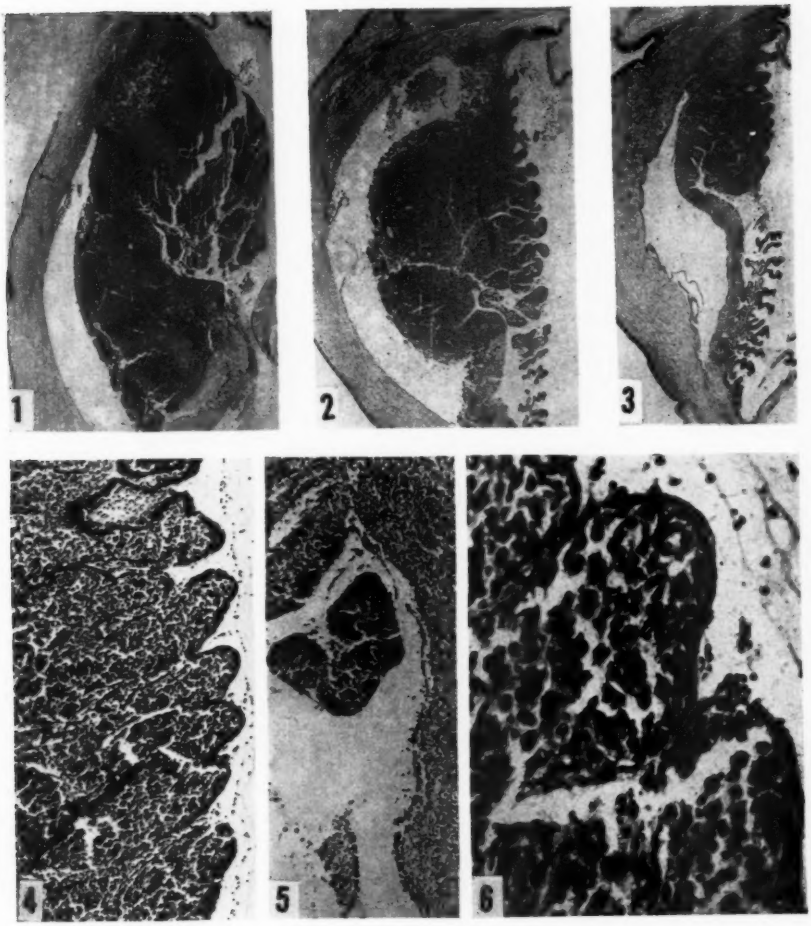
FIGURE 2.—No. 1980. The tumor tissue in the anterior chamber exceeds one low-power field (1:100) in size: 3 plus. × 13.

FIGURE 3.—No. 1126. The tumor tissue is about the size of one half of one low-power field (1:100): 2 plus. × 13.

FIGURE 4.—No. 1126. Same as fig. 3. × 62.

FIGURE 5.—No. 1808. The amount of vascularized tumor tissue is very small: 1 plus. × 62.

FIGURE 6.—No. 1126. This photograph demonstrates the relation of the tumor cells to the iris. × 248.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AN EVALUATION OF THE ANTIPROTEOLYTIC REACTION OF SERUM AS A TEST FOR MALIGNANT NEOPLASIA [1] [2] [3]

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The discovery of a specific test for the presence of a malignant neoplasm within the body would revolutionize our concepts of early diagnosis of "cancer." Such a positive test would at least distinguish those patients in whom a complete diagnostic work-up or continued observation with repeated studies would result in a positive diagnosis, while a negative test would, for practical purposes, exclude the diagnosis of malignant tumor. The patient with symptoms and signs of a lesion in a specific organ without sufficient diagnostic proof by our present methods could be subjected to treatment early on the evidence of such a test. Our total knowledge of malignant neoplasia would be rapidly extended, as was the knowledge concerning syphilis after discovery of the Wassermann reaction.

The search for such a specific test has been painstaking, but apparently unsuccessful to the present. In this search many tests have been developed, some of which originally have been considered specific. A few have been studied exhaustively, others only briefly; but in the final analysis all studied sufficiently have been shown to be nonspecific, subject to variable, but significant ratios of false negative and false positive tests. Practically all have been abandoned for general use after a trial because of this nonspecificity.

Those tests that have been carefully analyzed have at least one thing in common: the diseases that give false positive reactions. These usually include acute infections such as pneumonia, tuberculosis, pregnancy, post-traumatic states, and, less commonly, other generalized diseases such as diabetes, hyperthyroidism, and others. It will also be observed that most such suggested tests have had, as their basis, changes in the serum or plasma proteins, blood enzymes or antienzymes and their effects on coagulation of blood serum or plasma, or the breakdown of blood elements.

It is of no consequence to attempt review of all tests suggested, especially since excellent reviews such as those by Davidsohn in 1934 (*1*), Homburger (*2*) and Huggins (*3*) have already been presented. Many

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[3] The Tumor Clinic and the Tumor Registry of the Yale University School of Medicine, supported by the Jane Coffin Childs Fund and the Connecticut Cancer Society, were employed in the study.

[4] Much of this study was performed during tenure as a Senior Fellow of the American Cancer Society.

tests have been devised during the past few years. Those of Black *et al.* (*4, 5*) and Huggins *et al* (*6*) have perhaps attracted most attention (*7–24*).

Another nonspecific test, first reported by Brieger and Trebing (*25*), was developed on the basis of the antiproteolytic reaction of serum, a reaction first reported by Hildebrandt in 1893. Brieger and Trebing in 1908 reported that the reaction was positive (increased) in 95 percent of patients with "cancer" (*25*). Other investigators found an increase of the reaction in 70 to 92 percent of patients with "cancer" (*26, 27, 28*). It was soon discovered, however, that there was also an increase in the reaction in patients with many other diseases, including among others tuberculosis (*29*), acute infections (*30, 31, 32*), Graves' disease (*33*), diabetes (*35*), pregnancy (*35*), and many other diseases (*36*). Brieger (*37*) himself then called it a cachexia reaction, indicating that it occurred in diseases that caused cachexia.

After the work of Weil (*38*), who gave a complete review of the problem, this reaction was discarded as a test for malignant disease, primarily because of the subjectivity of the reaction as used at that time and the frequent false positive reactions. Since that time interest in the reaction has centered around its part in the clotting mechanism of blood, the control of infections, and its relation to proteolytic enzymes. An excellent review of the literature to 1943 has been furnished by Grob (*39*).

In the course of this work more objective methods for the determination of the antiproteolytic activity of the serum were developed, including two developed in this laboratory (*40–44*). The method, used in all cases to be reported in this paper, was reported in detail in a preliminary report (*40*). With a more objective method for performing the test and with the improvement of diagnostic methods and tests for other diseases that give false positive reactions, a re-evaluation of the reaction as a test for the presence of a malignant tumor was undertaken. Despite deliberate weighting of factors adverse to the reaction, an encouraging percentage of correct results has been obtained. Some interesting observations were made, and a few definite conclusions were reached. Since all so-called cancer tests proposed to date are nonspecific in nature, the information gathered may be of significance in evaluating other tests.

MATERIALS AND METHODS

Blood for examination was accepted without question and without information other than the name, number, and location of the patient necessary for proper follow-up, from all services of the hospital. Tests were also performed on each new admission to the tumor-detection clinic. No attempt was made to control the conditions under which the blood was drawn, and the tests were made on patients receiving all types of treatment. In as many patients as possible repeat tests were obtained, and in some few cases tests were repeated many times over a period of months. Such repetitions were made most frequently in those patients in whom the reaction obtained was not the one expected.

In addition, blood samples were specially requested on patients with disease states known to produce false positive and negative reactions. In this way large numbers of false positive and negative tests were obtained that would not have been observed if only patients with a question of malignant tumor had been tested. Such groups include pregnant individuals, postoperative patients, patients with tuberculosis, liver disease, and advanced or very early carcinomata. All patients have been followed as carefully as possible through routine and special visits to physician and clinic, and all charts have been studied carefully and repeatedly in order to obtain the maximum accuracy in diagnosis. The diagnosis in 98 percent of the cases of cancer has been proved pathologically; in the remaining 2 percent it was made by obvious clinical course, with continued observation, usually to death.

Between October 1947 and September 1, 1949, over 7,000 blood samples were obtained on 2,524 patients. Ages ranged from 2 years to 90 years, with the great majority (85 percent) being over 30 years of age. The age distribution of the patients with malignant tumor was essentially the same as that for patients without malignant tumor, although no statistical study of this factor was made. Of the 2,524 patients on whom tests were performed, 2,321 had adequate diagnostic studies and follow-up to permit a reasonably certain clinical diagnosis, while 203 patients were lost to follow-up, refused sufficient study or follow-up, or died without a definite diagnosis. In 85 of these 203 patients the diagnosis of malignant tumor was seriously considered.

In order to conform to the most rigid requirements of a test for the discovery of malignant neoplasm, the data to be reported include only the initial tests, although certain general observations and specific instances of cases with more than one test are used for purposes of discussion.

The results are reported as the ratio of the antiproteolytic titer of the unknown to that of a normal control serum (*40*). Under ordinary circumstances the serum from the normal patient shows a final clot in the 1:80 dilution, although occasionally the end dilution will be 1:70 or 1:90. The titer is then given as 80, 70, or 90, and the tested samples are reported as the percentage of the normal titer. Thus, if the control be 80, and the tested sample 120, this would be reported as 150 percent of normal. Using the ratios thus obtained, a statistical analysis was made of the first tests obtained in 2,213 cases and of 3,698 repeated tests in this same group of patients (table 1). The doubtful range, using individual cases, fell between 114 and 128 percent; and using the total 3,698 tests, the range was narrowed to 125 to 130 percent. This analysis is reported in detail (table 1).[5]

This analysis was used as the basis for classification of results as follows: negative 115 percent, doubtful 115–130 percent, positive all above 130 percent.

[5] Performed by Mrs. Sylvia Johnson, Statistician to the Departments of Public Health and Oncology, Yale University School of Medicine.

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TABLE 1.—*Preliminary statistical study*

Diagnoses	Initial test				All tests			
	Number	Range	[1] $\bar{x}$	[2] T±	Number	Range	[1] $\bar{x}$	[2] T±
All cases	2, 213	50–300	118. 5	19. 4	3, 698	40–314	132. 0	41. 6
Malignant	487	66–300	156. 4	26. 6	1, 113	40–314	163. 9	44. 7
Nonmalignant	1, 586	50–263	114. 2	17. 3	2, 439	50–275	117. 3	30. 3
Unclassified	140	75–271	141. 3	36. 1	146	75–286	138. 5	43. 7
MALIGNANT								
Primary	309	66–300	159. 4	27. 6	637	56–300	167. 1	42. 3
With metastasis	130	85–286	150. 8	38. 3	319	40–314	162. 9	50. 8
With recurrence	39	100–220	149. 9	37. 3	140	87–260	151. 8	37. 7
Basal cell	9	100–138	117. 8	20. 4				
NONMALIGNANT								
No apparent disease	354	50–185	101. 0	24. 9	401	50–187	103. 6	15. 7
Acute infection	113	85–263	153. 4	27. 1	151	85–275	161. 3	39. 2
Tuberculosis	101	62–250	138. 6	29. 5	139	62–250	135. 4	41. 5
Chronic infection	40	85–157	108. 7	20. 0	61	75–225	121. 5	31. 8
Pregnancy	43	85–213	155. 3	20. 9	46	85–213	164. 7	32. 2
Other disease	450	50–200	112. 2	17. 9	854	50–241	114. 7	23. 8
Benign	319	50–185	103. 0	13. 1	391	50–186	105. 4	17. 0
No recurrence	135	70–200	110. 0	18. 2	331	70–200	110. 4	20. 5
Postoperative	30	75–240	155. 5	42. 9	65	75–273	163. 6	37. 6

C. L.[3]	Nonmalignant	Malignant	Total	[4] Z	C. L.[3]	Nonmalignant	Malignant	Total	[4] Z
	Percent	*Percent*	*Percent*			*Percent*	*Percent*	*Percent*	
Upper	70. 5	24. 0	94. 5	114	Upper	75. 5	24. 8	100. 3	125
Lower	78. 5	24. 1	102. 6	128	Lower	77. 0	22. 6	99. 6	130
	Doubtful range: 114–128					Doubtful range: 125–130			

[1] $\bar{x}$=mean.
[2] T=mean deviation limit.
[3] C. L.=confidence limits.
[4] Z=range.

RESULTS

Table 2 lists the results obtained with the total group of 2,524 cases. As previously suggested (*40*) the sera from patients with carcinoma without demonstrable metastases are positive in a higher percentage of cases, 69.6 percent, than are those with metastases, 63 percent. It can also be seen that the sera from patients with sarcoma and lymphoma give a higher percentage of positive reactions (70 and 79.3 percent). This differential between groups has been consistent with each re-evaluation from the first, with 463 cases (*40*), through 5 subsequent evaluations (not published), with increasing numbers of cases, to the present report. Only 2 of the 29 patients with lymphoma gave negative reactions, and both of these were in cases of macrofollicular lymphoma with doubtful

clinical activity. Of the 540 patients with malignant tumors, 368, or 68.1 percent, gave positive reactions and 63, or 11.6 percent, gave doubtful reactions for a total of 431 cases, or 79.7 percent positive and doubtful reactions as compared with 109, or 20.2 percent negative reactions. Of 169 patients who had had definitive treatment of a malignant tumor, 29 had definite recurrences and 140 were without evidence of recurrence. Of the 29 with recurrence, 20 (68.9 percent) gave a positive test, 1 (3.6 percent) a doubtful test, and 8 (27.5 percent) a negative test; whereas of the 140 without evidence of recurrence only 13 (9.2 percent) gave a positive test, 10 (7.1 percent) a doubtful test, and 117 (83.5 percent) a negative test.

TABLE 2

	Total number	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma without metastases	297	207	69.6	35	11.8	55	18.5
Carcinoma with metastases	165	104	63.0	22	13.3	39	23.6
Carcinoma recurrent	29	20	68.9	1	3.6	8	27.5
All carcinoma	491	331	67.4	58	11.1	102	20.8
Sarcoma including mycosis fungoides	20	14	70.0	1	5.0	5	25.0
Lymphoma	29	23	79.3	4	13.7	2	6.9
All with malignant tumor	540	368	68.1	63	11.6	109	20.2
Leukemia	9	4	44.4	2	22.2	3	33.3
Basal cell tumor	6	2	33.3	1	16.6	3	50.0
Malignant tumor treated without evidence of recurrence	140	13	9.2	10	7.1	117	83.5
Benign tumor	283	16	5.6	20	7.0	247	87.0
No apparent disease	388	13	3.3	25	6.4	350	90.2
Peptic ulcer	61	14	23.0	11	18.0	36	59.0
Acute infection	116	91	78.5	10	8.6	15	12.9
Chronic infection	165	16	9.7	12	7.3	137	83.0
Pregnancy	51	43	84.3	3	5.9	5	9.8
Tuberculosis	108	52	48.1	15	13.9	41	38.0
Liver disease	16	7	43.8	5	31.1	4	25.0
Post-operative	31	27	87.0	2	6.5	2	6.5
All other diseases	407	58	14.3	50	12.3	299	73.4
All patients without malignant tumor	1,626	337	20.7	153	9.3	1,136	70.0
? Malignant tumor (unproved)	85	45	52.9	9	10.6	31	36.4
Inadequate follow-up	118						

In contrast, of 1,626 patients with no disease or disease other than malignant tumor, only 20.7 percent gave positive reactions, 9.4 percent gave doubtful reactions and 69.8 percent gave negative reactions. Reference to table 2 indicates that the highest percentage of false positive reactions occurs in patients with acute infections, pregnancy, tuberculosis, liver disease, and in the postoperative state, while patients with chronic infections, peptic ulcer, and all other diseases give occasional false positive reactions, and patients without apparent disease or with benign tumors rarely give positive reactions (3.3 and 5.6 percent, respectively). Eight of the 16 patients with benign tumor who had a positive

reaction had repeated tests, and in 7 this test was negative the second time, while in 1 it was doubtful. Ten of the 13 patients without disease had repeated tests and only 2 gave a positive test the second time and 1 of these patients subsequently had 2 negative tests, while the other remained positive. In contrast, patients with malignant tumor with a primary positive reaction gave repeat positive tests in over 95 percent of the cases.

From these data it seems clear that in the presence of acute infection, pregnancy, active tuberculosis, liver disease, and in the postoperative state, the test has no significance. Fortunately most of these diseases are easily diagnosed by adequate methods at our disposal, so that they do not pose a problem in so far as the detection of malignancy is concerned. With the acute infections and postoperative state the serum antiproteolytic titer returns to normal in all cases within 4 weeks and in many cases in less than 2 weeks after the subsidence of infection or with wound healing. With pregnancy the titer returns to normal 2 to 4 weeks after delivery.

TABLE 3.—*Cases with diagnosis "Question of malignant tumor"*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Malignant tumor	240	182	75.8	25	10.4	33	13.7
No malignant tumor	287	42	14.6	36	12.5	209	72.8

In order to evaluate the reaction more accurately for diagnostic significance, the cases in which the possibility of malignant tumor was entertained as a preliminary diagnosis or in which the preliminary diagnosis was wrong, at the time the test was made, were separately studied. A group of 527 cases was collected, of which 240 were eventually proved to have malignant neoplasia and 287 were found to be free of such tumor (table 3). This would indicate that in this group the preliminary diagnosis was approximately 50 percent accurate. Of the 240 patients eventually proved to have malignant tumors, 182 (75.8 percent) gave positive tests, 25 (10.4 percent) gave a doubtful test, and only 33 (13.8 percent) gave a negative test, while of the 287 patients proved to be without malignant tumor, only 42 (14.6 percent) gave a positive, 36 (12.5 percent) a doubtful, and 209 (72.8 percent) a negative test.

Another group, for comparison, includes those patients seen in tumor or detection clinic (table 4). This is a method of screening out the

acutely or seriously ill patients and most of the pregnant individuals. In this group, the patients with malignant tumors showed ratios practically identical with the total group: 67.1 percent positive reactions, 9.1 percent doubtful reactions and 23.6 percent negative reactions. The data for the patients without malignant tumor in this group are significantly better than in the total group without malignant tumor, since only 47 (5.1 percent) of 911 patients gave a positive reaction, 76 (8.4 percent) a doubtful reaction, and 788 (86.5 percent) gave a negative reaction. It will also be observed that no pregnant patient (only 4 cases) gave a positive reaction, since all were under 3 months' pregnant. It has been confirmed that the reaction becomes positive some time during the second month of pregnancy. Also, only 1 of 14 patients seen in tumor clinic with a diagnosis of tuberculosis gave a positive reaction.

TABLE 4.—*Patients seen in tumor clinic*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Malignant tumor	131	88	67.1	1	9.1	31	23.6
Treated malignant tumor without evidence of recurrence	124	10	8.0	9	7.2	105	84.6
Benign tumor	197	7	3.5	15	7.6	175	88.8
No apparent disease	356	13	3.6	19	5.3	324	91.0
Tuberculosis	14	1	7.1	1	7.1	12	85.7
Pregnancy	4	0	0	1	25.0	3	75.0
All other diseases	340	26	7.6	40	11.7	274	80.6
Total without malignant tumor	911	47	5.1	76	8.4	788	86.5
Doubtful diagnosis	43	18	41.8	9	21.0	16	37.2

These results are not sufficiently correct to indicate a definitive value in the diagnosis of malignant disease. They are, however, sufficiently accurate to suggest a real value of the reaction as a supplementary test in certain cases of suspected malignant disease or to suggest more thorough study in patients who give a positive reaction though apparently well.

A study of the lesions of different organ systems was undertaken. A detailed report on lesions of the stomach and duodenum has been published (*45*). Cases have been added to this group (table 7), and, in addition, a careful analysis of diseases of the lung (table 5), pleura and mediastinum (table 6), kidney and ureter (table 8), breast (table 9), female pelvic organs (table 10), biliary tract and pancreas (table 11), mouth, tongue, and larynx (table 12), prostate (table 13), esophagus (table 14), thyroid gland (table 15), bladder (table 16), bone and joint (table 17), colon (table 18), and skin, lip, and subcutaneous tissues (table 19).

TABLE 5.—*Lung lesions*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma	97	90	92.8	3	3.1	4	4.1
Treated carcinoma without evidence recurrence	5	0	0	0	0	5	100.0
Tuberculosis	66	27	40.9	9	13.6	30	45.4
Acute infection	26	18	69.2	2	7.6	6	23.1
Chronic infection	27	4	14.7	0	0	23	85.2
Benign tumor including cyst	10	3	30.0	1	10.0	6	60.0
All without carcinoma	134	54	38.7	16	8.9	73	52.2
Doubtful	10	2	20.0	4	40.0	4	40.0

TABLE 6.—*Pleura and mediastinum*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Malignant	11	8	72.8	2	18.1	1	9.0
No evidence recurrence	2	1	50.0	0	0	1	50.0
Benign	7	0	0	0	0	7	100.0
Infection	2	2	100.0	0	0	0	0
Tuberculosis	4	2	50.0	2	50.0	0	0
All others	11	0	0	0	0	11	100.0
Total without malignancy	24	4	16.6	2	8.3	18	75.0

TABLE 7.—*Stomach*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma without metastasis	10	8	80.0	2	20.0	0	0
Carcinoma with metastasis	16	11	68.7	3	18.8	2	12.5
All primary carcinoma	26	19	73.0	5	19.2	2	7.7
Recurrent carcinoma	4	2	50.0	1	25.0	1	25.0
All carcinoma	30	21	70.0	6	20.0	3	10.0
No evidence of recurrence	12	0	0	0	0	12	100.0
Peptic ulcer with hemorrhage	13	10	77.0	1	7.7	2	15.4
Peptic ulcer, perforating	3	2	66.6	1	33.3	0	0
Peptic ulcer, uncomplicated	45	2	4.4	9	20.0	34	75.6
Gastritis	1	0	0	0	0	1	100.0
Gastritis and hemorrhage	2	1	50.0	1	50.0	0	0
All others	11	0	0	2	18.2	9	81.8
All without carcinoma	67	10	14.9	14	20.9	43	64.2

TABLE 8.—*Kidney and ureter*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma, primary	10	9	90.0	1	10.0	0	0
Carcinoma, recurrent	4	3	75.0	0	0	1	25.0
Total with carcinoma	14	12	85.7	1	7.1	1	7.1
No evidence recurrence	1	0	0	0	0	1	100.0
Infection	17	11	64.8	1	5.9	5	29.2
Calculus	8	1	12.5	2	25.0	5	62.5
Benign	7	0	0	0	0	7	100.0
Tuberculosis	1	1	100.0	0	0	0	0
Total without carcinoma	33	13	39.5	3	9.2	17	51.5

TABLE 9.—*Breast*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma without metastasis	20	5	25.0	2	10.0	13	65.0
Carcinoma with metastasis	21	9	42.8	2	9.5	10	47.6
Carcinoma, recurrent	12	7	58.3	0	0	5	41.6
Sarcoma	2	1	50.0	0	0	1	50.0
Total with malignancy	55	22	40.0	4	7.3	29	52.7
Benign	75	3	4.0	7	9.3	65	86.6
Infection	6	2	33.3	0	0	4	66.6
No evidence of recurrence	27	1	3.7	1	3.7	25	92.6
Total without malignancy	108	6	5.6	8	7.4	94	87.0

TABLE 10.—*Female pelvic organs including cervix*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma of cervix	16	8	50.0	2	12.5	6	37.5
Carcinoma of uterus	3	2	66.6	1	33.3	0	0
Carcinoma of ovary	3	2	66.6	1	33.3	0	0
All primary carcinoma	22	12	54.5	4	18.1	6	27.2
Recurrent carcinoma	10	5	50.0	1	10.0	4	40.0
All carcinoma	31	17	54.8	4	12.9	10	32.2
No evidence of recurrence	5	0	0	0	0	5	100.0
Cervicitis	17	3	17.6	8	47.1	6	35.3
Benign	24	1	4.2	0	0	23	95.8
Acute inflammatory	3	3	100.0	0	0	0	0
All others	18	0	0	2	11.1	16	88.9
Endometriosis	1	1	100.0	0	0	0	0
All without carcinoma	117	8	6.7	10	8.5	99	84.6

TABLE 11.—*Biliary tract and pancreas*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma liver	2	1	50.0	1	50.0	0	0
Carcinoma common duct	1	1	100.0	0	0	0	0
Carcinoma pancreas	10	9	90.0	1	10.0	0	0
All carcinoma	12	11	91.6	1	8.4	0	0
No evidence recurrence	1	0	0	0	0	1	100.0
Jaundice—serum	2	0	0	2	100.0	0	0
Jaundice—hepatitis infection	4	1	25.0	1	25.0	2	50.0
Jaundice—obstructive	4	3	75.0	1	25.0	0	0
Benign tumor	2	0	0	0	0	2	100.0
Acute cholecystitis	4	4	100.0	0	0	0	0
Chronic cholecystitis	17	0	0	0	0	17	100.0
Acute pancreatitis	1	1	100.0	0	0	0	0
Chronic pancreatitis	1	0	0	0	0	1	100.0
Cholangitis	1	1	100.0	0	0	0	0
Cirrhosis	7	4	57.2	3	42.8	0	0
All without carcinoma	44	14	31.8	7	15.9	23	52.2

TABLE 12.—*Mouth, tongue, and larynx*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma, primary	28	19	68.0	5	17.8	4	14.2
Carcinoma, recurrent	6	1	16.7	3	50.0	2	33.3
Benign	9	0	0	1	11.1	8	88.9
Inflammatory	7	0	0	0	0	7	100.0
No evidence recurrence	8	1	12.5	0	0	7	87.5
Leukoplakia	12	3	25.0	1	8.3	8	66.7
No carcinoma	28	3	10.7	2	7.1	23	82.1
Doubtful	7	7	100.0	0	0	0	0

TABLE 13.—*Prostate*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma, prostate	27	21	77.7	3	11.1	3	11.1
Carcinoma, prostate, treated	1	0	0	0	0	1	100.0
Benign overgrowth prostate	23	4	18.1	4	13.1	15	68.1
Prostatitis	1	0	0	0	0	1	100.0
Bladder neck obstruction	5	0	0	0	0	5	100.0
All not carcinoma	29	4	13.8	4	13.8	21	72.4
Unproved	3	2	66.6	0	0	1	33.3

TABLE 14.—*Esophagus*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma, primary	37	21	55.5	5	13.9	11	30.5
Carcinoma, recurrent	1	0	0	0	0	1	100.0
Esophagitis	1	0	0	1	100.0	0	0
Cardiospasm	2	1	50.0	0	0	1	50.0

TABLE 15.—*Thyroid gland*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma	4	3	75.0	0	0	1	25.0
No evidence recurrence, carcinoma	2	0	0	0	0	2	100.0
Benign	26	2	7.6	3	11.5	21	80.7
Inflammatory	2	0	0	0	0	2	100.0
Hyperthyroid	13	5	38.4	0	0	8	61.5
Hypothyroid	4	0	0	0	0	4	100.0
Branchial and thyroglossal cysts	1	0	0	0	0	1	100.0
All without carcinoma	46	7	15.2	3	6.5	36	78.4

TABLE 16.—*Bladder*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma	5	4	80.0	0	0	1	20.0
No evidence recurrence	1	0	0	0	0	1	100.0
Papilloma	4	1	25.0	1	25.0	2	50.0
Infection	4	1	25.0	1	25.0	2	50.0
All others	3	0	0	0	0	3	100.0
All without carcinoma	11	2	18.1	2	18.1	7	63.6

TABLE 17.—*Bone and Joint*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Primary malignancy	3	2	66.6	0	0	1	33.3
Metastatic malignancy	3	3	100.0	0	0	0	0
Total with malignancy	6	5	83.3	0	0	1	16.6
No evidence recurrence malignancy	3	0	0	0	0	3	100.0
Benign	6	1	16.6	0	0	5	83.3
Osteo-arthritis and Marie Strumpell's disease	5	0	0	0	0	5	100.0
Rheumatoid arthritis	2	0	0	1	50.0	1	50.0
Infection	3	1	33.3	1	33.3	1	33.3
Tuberculosis	1	1	100.0	0	0	0	0
Mechanical	1	0	0	1	100.0	0	0
Fracture	2	1	50.0	1	50.0	0	0
Total without carcinoma	20	4	20.0	4	20.0	12	60.0

TABLE 18.—*Colon*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma without metastasis	29	22	75.9	4	13.8	3	10.3
Carcinoma with metastasis	13	3	23.1	3	23.1	7	53.8
Carcinoma, recurrent	5	5	100.0	0	0	0	0
Total with carcinoma	47	30	63.8	7	15.0	10	21.2
Carcinoma without evidence recurrence	23	1	4.3	1	4.3	21	91.4
Diverticulitis and acute infection	5	1	20.0	1	20.0	3	60.0
Colitis and proctitis	9	1	11.1	1	11.1	7	77.8
Diverticulosis, etc	6	0	0	0	0	6	100.0
Benign and polyps	11	0	0	0	0	11	100.0
All others	20	2	10.0	1	5.0	17	85.0
Total without carcinoma	74	5	6.7	4	5.4	65	87.8

The cases of *primary* malignant tumor with or without metastases have been tabulated in table 20. The test is positive most frequently in patients with malignant tumor in the lung, 92.8 percent; biliary tract and pancreas, 91.6 percent; kidney, 90 percent; bone and joint, 83.3 percent; bladder, 80 percent; prostate, 77.7 percent; thyroid gland, 75 percent; stomach, 73 percent; pleura and mediastinum, 72.8 percent; mouth, tongue, and larynx, 68 percent; and uterus and ovary, 66.6 percent. In patients with malignant lesions of the colon it is positive in 59.5 percent; esophagus, 55.5 percent; cervix, 50 percent; breast, 34.1 percent, and skin and lip, 27.5 percent (table 20). The lesions with which the reaction is positive in the smallest percentage of cases are those in which the diagnosis is made relatively easily and early by other methods at our disposal.

TABLE 19.—*Skin, lip, and subcutaneous tissues*

	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Carcinoma skin: ≤1 cm	8	0	0	3	37.4	5	62.6
Carcinoma skin: >1 cm	9	6	66.6	2	22.2	1	11.2
Basal cell skin: ≤1 cm	7	0	0	2	28.5	5	71.4
Basal cell skin: >1 cm	0	0	0	0	0	0	0
Carcinoma lip: ≤1 cm	7	0	0	0	0	7	100.0
Carcinoma lip: >1 cm	4	3	75.0	0	0	1	25.0
Malignant: soft tissues	4	1	25.0	1	25.0	2	50.0
Mycosis fungoides	1	1	100.0	0	0	0	0
Total carcinoma	40	11	27.5	8	20.0	21	52.5
Benign skin	26	1	3.8	0	0	25	96.2
Benign subcutaneous	11	0	0	0	0	11	100.0
Ulcer skin	1	0	0	0	0	1	100.0
Hyperkeratosis, Bowens, and all others	11	0	0	1	9.1	10	90.9
Benign lip	1	0	0	0	0	1	100.0
Melanoma	1	0	0	0	0	1	100.0
Benign skin and subcutaneous	50	1	2.0	1	2.0	48	96.0

Certain types of malignant tumor of superficial sites, such as the skin, breast, and cervix, failed to give positive reactions in a high percentage of cases. It is a reasonable supposition that the size of the lesion must be of importance in the amount of reaction produced, since a tiny lesion would

TABLE 20.—*Primary malignant tumors**

Organ system	Total cases	Positive		Doubtful		Negative	
		Number	Percent	Number	Percent	Number	Percent
Lung	97	90	92.8	3	3.1	4	4.1
Biliary tract	13	11	91.6	2	8.4	0	0
Kidney and ureter	10	9	90	1	10.0	0	0
Bone and joint	6	5	83.3	0	0	1	16.7
Bladder	5	4	80	0	0	1	20.0
Prostate	27	21	77.7	3	11.1	3	11.1
Thyroid	4	3	75	0	0	1	25.0
Stomach	26	19	73	5	19.2	2	7.7
Pleura and mediastinum	11	8	72.8	2	18.1	1	9.0
Mouth, tongue, and larynx	28	19	68	5	17.8	4	14.2
Uterus and ovary	6	4	66.6	2	33.3	0	0
Colon	42	25	59.5	7	16.6	10	23.8
Esophagus	37	21	55.5	5	13.9	11	30.5
Cervix	16	8	50	2	12.5	6	37.5
Breast	41	14	34.1	4	9.7	23	56.1
Skin, lip, and subcutaneous	40	11	27.5	8	20.0	21	52.5
Total	409	272	66.5	49	12.0	88	21.5

*With or without metastases. Recurrent tumors not included. This table does not include all cases from table 2.

not be expected to give a definite reaction as would a large lesion. This explanation is confirmed by table 19, where the cases are broken down into lesions less than 1 cm. and more than 1 cm. in diameter. No patient with a tumor of the skin less than 1 cm. in diameter gave a positive reaction, whereas 66.6 percent of the epidermoid carcinomata of the skin and 75 percent of the carcinomata of the lip over 1 cm. in diameter gave a positive reaction.

This is substantiated in part by the fact that among the female pelvic organs, (table 9) the total low figure of 54.5 percent positive tests was due to the presence of numerous cervical lesions, several of which were very small and two of which were found only because of a positive Papanicolaou test, while the ovarian and uterine malignant tumors each gave positive tests in 66.6 percent of cases.

Other factors to be considered such as infection, may be important in certain organ systems, especially since infection itself may produce a positive reaction. The high percentage of positive reactions in carcinoma of the lung might be due to superimposed infection. This possibility is counteracted by the fact that lesions of the colon, which are almost invariably infected, give a much lower percentage of positive reactions than do those in the lung, kidney, prostate, and bone, while the cervical lesions give extremely low percentages even in the presence of infection. Furthermore, if one removes from the pulmonary group all patients with carcinoma of the lung with a fever over 100 degrees or a white blood-cell count over 12,000 the ratio of positive reactions remains over 85 percent.

Jaundice may be of importance in patients with carcinoma of the pancreas and common ducts because there are indications that obstructive jaundice causes an elevation of the antiproteolytic titer. However, patients with carcinoma of these organs without jaundice have elevated titers as frequently as do those with jaundice.

Cachexia has been implicated as the cause of this reaction (*37*). This opinion was opposed by von Bergmann and Meyer (*28*), and in this series patients with evident distant metastases, who are more likely to be cachectic, had a lower ratio of positive reactions. In addition, practically every patient in whom cachexia was a notable feature of the disease, even if they did not have definite metastases, gave a negative reaction rather than a positive reaction.

It has been maintained by many authors that the reaction is due to the breakdown products produced by degeneration of the tumor and that it is elevated only when necrosis is present in the tumor (*46*). This has not been confirmed by our clinical studies, where many small actively growing tumors with minimal or no necrosis gave positive reactions while some of the patients with the largest sloughing necrotic tumors gave negative reactions. In addition, mice with spontaneous breast tumors show less reaction with large necrotic tumors than with smaller actively growing tumors (unpublished data).

DISCUSSION

The serum antiproteolytic reaction, and most tests for the diagnosis of malignant tumors described to date, are not of sufficient specificity to be considered diagnostic in any given case. However, if certain limitations are recognized, the reaction can be of definite value as a screening or detection method or as a confirmatory reaction. In the presence of acute infection, pregnancy, and in the postoperative state there is no value to be gained by performance of the test. In cases of active tuberculosis and chronic infections of large areas, such as advanced bronchiectasis, a positive reaction can not be considered evidence for the presence of a malignant tumor. If a tumor is small, a negative test does not exclude the presence of cancer, and, with only 67 percent positive tests, a negative test should never lead one to forfeit complete diagnostic studies where indicated.

There are times in clinical practice, however, when the results of the reaction might lead one to a definite course of action. In poor-risk patients with a presumptive clinical diagnosis of carcinoma in a major viscus or organ system, such as the lung or biliary tract where the test is positive in a very high percentage of cases and where the mortality of exploratory surgery is high, a negative reaction might contraindicate major surgery, while a positive test would lead one to attempt surgery, since the chance of error would not exceed and might be less than the expected operative mortality risk.

In a supposedly well individual a positive reaction should be considered as a warning reaction and if on repetition it remains positive, that patient should receive a complete work-up. Rarely will such a patient be found free of disease. In patients with benign tumor and other diseases that seldom give false positive reactions, a continued positive reaction should indicate a very complete work-up or follow-up, or both. The chance is definite that such patients will have carcinoma.

The false negative reactions in malignant tumors of internal organs are less important than seem indicated in this report, since many have subsequent doubtful or positive reactions, while those with repeated negative reactions frequently are found to have metastatic disease.

SUMMARY

Results of serum antiproteolytic tests on 2,524 patients are reported. Positive tests were obtained in a high percentage (68.1 percent) of patients with a malignant tumor. False positive tests were frequently obtained in patients with acute infections, pregnancy, tuberculosis, liver disease, and in the postoperative state. False positive reactions were seldom obtained in patients without apparent disease (3.3 percent) or with benign tumors (5.6 percent). An analysis of the patients with diseases in the various organ systems revealed that carcinoma of the lung gave the highest

ratio of positive tests (92.8 percent). Malignant tumors of other internal organs also gave high ratios of positive tests: biliary tract and pancreas 91.6 percent, kidney 90 percent, bladder 80 percent, prostate 77.7 percent, thyroid 75 percent, stomach 73 percent, and pleura and mediastinum 72.8 percent.

Lacking a specific test for malignant neoplasia, a nonspecific reaction of this type may be of value as a detection method to suggest the presence of internal malignant tumors, or as a confirmatory diagnostic test.

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AN INDUCED ADAPTATION IN A TRANSPLANTABLE TUMOR OF MICE

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When a tumor is transferred to a new host a very complex system of reactions is brought into play which undoubtedly comprises many unknown factors. However, it may be assumed that a practical expression of the effective sum of these factors is seen by observing whether the graft takes and whether it grows progressively or regresses.

Among the factors that control or strongly influence the outcome of transplantation of a tumor is the genetic relationship of the tumor to the host. This relationship seems at present to be the primary element in this complex situation. Extensive work on this aspect, begun by Loeb and extended by Tyzzer, Little, Strong, Bittner and others has led to concepts that are sometimes referred to as the "genetic laws of transplantation." An excellent summary of this field has been written by Little (*1*) and references to detailed papers on the subject may be found in his bibliography.

As background for the work to be presented herein it will suffice to recall only the following genetic principles. A tumor derived from a mouse of an inbred strain will grow in all mice of the same strain and in all F_1 hybrids having that inbred strain as one parent. It will not grow in other strains or in other F_1 hybrids except for certain exceptional and rarely occurring tumors. In resistant backcross animals, *i. e.*, animals derived by backcrossing susceptible F_1 hybrids (strain of origin of the tumor $\times$ resistant strain) to resistant strain, the percentage of successful grafts is usually low but may vary from 50 percent to 0.002 percent depending on whether 1 or 15 genetically controlled "factors" are required by the particular tumor used. No consideration is given here to the well known but unusual tumors such as Sa 37, Sa 180, and such whose hereditary makeup is entirely unknown and which appear to be in part specific and partly nonspecific depending upon circumstances that cannot be analyzed at the moment.

Spontaneous or induced tumors if transplanted will conform rather closely to the above principles. Upon continued transplantation some tumors have exhibited relatively sudden and permanent changes in transplantability which Strong (*2*, *3*) has called mutations. Bittner (*4*) and Cloudman (*5*) confirmed Strong's observations on tumors and MacDowell and his coworkers (*6*) reported that immunologic differences were found between leukemic cells in the original animals and those passed through a long series of hosts.

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The results reported in this communication indicate that the passage of tumors in varied hosts may induce changes in transplantability which appear to differ from previously reported mutations and to be more in the nature of an induced adaptation. Until more is known of both processes it is not possible to decide whether the difference is apparent or real. However that may be, it is possible, while maintaining the parent stock tumor unchanged, to produce at will daughter tumors that kill three times as many animals as would be expected on genetic grounds.

MATERIALS AND METHODS

The mice used as hosts were all of known genetic constitution and derived from stocks in the colony of Dr. W. E. Heston at the National Cancer Institute. Law has published a description of the strains used (*7*). The hosts include three categories; (*a*) strain C3H, (*b*) reciprocal F_1 hybrids between strains C3H and C (B alb C), and (*c*) resistant backcross animals derived by mating F_1 hybrid females to C males. Both of the reciprocal hybrids were used for the backcross so that two classes resulted, those backcross animals having strain C3H maternal grandmothers and those having C maternal grandmothers. This distinction was made because of previous work (*8*) and for reasons that will appear later.

Both sexes were used and distributed approximately equally. All hosts were between 8 and 12 weeks of age except for 3 small groups that were 1, 4, and 7 months old. The latter 3 groups gave the same results as the others.

The tumor, which is called C3HBA and has been described previously (*8*), is a mammary adenocarcinoma which arose spontaneously in a C3H mouse. It grows in all C3H mice but has never grown in a C mouse although many inoculations have been made. For this work material has been taken at frequent but irregular intervals between the 42d and 63d transplant generation of the tumor. The milk agent is present in the tumor. (Unpublished results of work done in collaboration with Dr. H. B. Andervont).

Using aseptic precautions, a brei of clean tumor tissue was prepared with the aid of a tissue press, diluted with an equal quantity of physiologic saline, and loaded into a tuberculin syringe carrying a 22-gauge needle. In all cases 0.025 cc. of the suspension was inoculated subcutaneously in the right inguinal region of the recipient. This dosage is sufficient to result in 100 percent takes in strain C3H animals and the F_1 hybrids.

The resulting tumors were measured at weekly intervals for 4 weeks and finally at death of the host. Animals without a tumor or in which a tumor had grown and regressed were observed until a few weeks after the last tumor-bearing animal in the group had died.

Small groups of animals (10 or less) were housed in plastic cages containing sawdust bedding and allowed free access to tap water and Purina laboratory chow. The animal room is air-conditioned and kept at a constant temperature of 78° F. and humidity of 30 to 40 percent.

Two conditions were imposed upon the transplanted tissue; these will be spoken of as the direct and the indirect method of transplantation.

In the direct transplantation, tissue was taken from stock tumors growing in C3H hosts and inoculated directly into backcross animals. These tumors had never been in any host other than strain C3H since the original tumor arose. In order to guard against the possibility that spontaneous mutation of the tumor during the course of the experiments might influence the results, the direct transfers were made in 16 separate experiments extending over a period of 14 months and covering approximately the same period as that represented by the indirect transfers. A total of 281 backcross hosts was used for the direct transfers.

Indirect transplantation was accomplished by transferring C3H tumor tissue first to F_1 hybrid hosts, allowing 3 weeks for growth and then using this tissue for inoculation of the backcross animals. It should be noted that in this method the tumor tissue originates in the same population of C3H hosts and finally grows in the same population of backcross hosts as in the direct method. It seems correct to say that by both methods the resulting tumors in backcross animals are composed of cells which are descendants of the same line of cells but with the single difference that there is a hybrid host intervening in the case of the indirect method. Here again precautions were taken to avoid mistakes due to spontaneous mutations of the tumor or effects produced by long residence in hybrid hosts. Twenty-one separate experiments were done by the indirect method. In 11 of these, the transfer was made through a hybrid of the C3H maternal line to a backcross recipient having the same maternal line, and in 10 experiments the hybrid and backcross hosts were of the C maternal line. In some cases the tumor had grown in a hybrid host for only 1 generation; in other cases the tissue had been serially transferred through hybrid hosts for varied numbers of generations up to 20.

RESULTS

The final results are known in 514 backcross mice which were inoculated with the tumor. Not all of these are included in the tabulation because 58 were killed within 5 weeks after inoculation. This was done before it was decided to follow all tumors until death of the animal. At that early stage of the work there were 20 "takes" among 58 backcross recipients of tumor taken from hybrid donors compared with 0 "takes" in 30 backcross recipients of tumor taken from strain C3H donors. This quantitative difference in "takes" is present, in varied degree, throughout the course of the experiments and would lead to the same conclusions as have been reached. However, it is possible to believe that comparison of "takes" is less convincing than comparison of tumors that grow progressively and kill the host; therefore only the latter are tabulated herein.

Table 1 shows the results in 456 backcross hosts that were observed until death with a progressively growing tumor or that failed to develop a tumor. The reciprocal hybrids and backcross animals are shown both separately and combined. This was done because in previous work (*8*) it was observed that there is a maternal influence on the growth rate of this tumor in F_1 hybrids. Although growth rate is not directly involved in this report

TABLE 1.—*Comparison of the percent of animals that died with a progressively growing tumor after inoculation with tumor taken from a C3H host or from a hybrid host*

Type of host	Source of tumor inoculum	Total mice inoculated	Number positive	Number negative	Percent positive	Statistical test	
						X^2	p
(C3HC × C) BC..	C3H	182	15	167	8.2	21.6	<0.001
	Hybrid (C3H × C) F_1..	86	26	60	30.2		
(CC3H × C) BC..	C3H	99	7	92	7.1	5.2	0.023
	Hybrid (C × C3H) F_1..	89	16	73	18.0		
Combined data both backcrosses.	C3H	281	22	259	7.8	23.3	<0.001
	F_1 Hybrid	175	42	133	24.0		

The significance of the difference is indicated by the values for Chi square and p shown. The authors are grateful to Mr. Jerome Cornfield for making the statistical analysis of the results.

it was thought best to keep the maternal lines separate. The data in all 3 categories show that approximately 3 times as many progressively growing tumors in backcross hosts resulted when the graft was taken from a hybrid donor as when it was taken from a pure C3H donor. Statistical analysis indicates that the difference is highly significant ($p=<0.001$).

Lack of space for breeding the animals necessitated the use of small groups in any one experiment. The data shown in the table represent the combined results in 16 separate groups of backcross recipients inoculated "directly" and 21 separate groups inoculated "indirectly." The populations represented by these groups can be assumed to be of the same genetic constitution but, as would be expected, the percentage of positive animals in a given group has a random distribution about a mean and therefore varied from group to group. However, the results taken group by group give the same impression as the combined data (*e. g.* "direct" transplantation in 2 groups resulted in 0 tumors in 10 animals and 2 tumors in 14 animals, whereas comparable groups in the "indirect" series had 3 tumors in 10 animals and 4 tumors in 10 animals). For that reason it is thought to be unnecessarily complicated to give the results in individual groups.

Nevertheless, 2 important facts were observed by inspecting the results in individual groups. The first is that during the 14 months in which tumor was taken 16 times from the parent stock (between the 42d and 63d generations of stock tumor) for transfer to backcross recipients there had not been a spontaneous mutation of the tumor such as might materially alter its transplantability into backcross hosts. In fact it so happened that in the last group of hosts in this category (direct transfer) a result below the average for the entire category was obtained. The second important fact to emerge from an inspection of the results in individual groups is that tumor tissue which was derived from the 20th generation of growth in hybrids did not differ appreciably in its transplantability to backcross hosts from that which had grown only 2 gener-

ations in hybrids. Transfers were not made to backcross recipients from every generation in hybrid but from various generations of 2 series. In these series the exact percentage of positive animals in a given group was distributed at random, and there was no evidence of a progressive change with increasing length of residence in hybrid hosts.

DISCUSSION

The results of these experiments clearly show that while growing in an F_1 hybrid between a susceptible and a resistant strain this tumor underwent some sort of physiologic change which enabled it to grow in an unexpectedly large number of resistant strain backcross animals, but the mechanism of the change is not evident. One can imagine a large number of factors that might be at work in these experiments, and more than one combination of circumstances could account for the phenomena observed. To select one interpretation from among those possible appears premature, but it should be worthwhile to weigh certain possibilities in explanation of the results obtained.

The field to which this work is related is a large one and, although there may be a certain amount of relevancy in some of the work previously reported on changes in the host, such changes are omitted from this discussion for the sake of brevity because in this work limits have been set by considering only final results in a uniform population of hosts and observing variations induced in the tumor. It also seems best to leave out of consideration those cases in which a tumor became widely or completely transplantable within a species, since these seem to be more closely related to spontaneous mutations.

A change in the potentiality for growth of a tumor similar to that described here has sometimes been ascribed to "increased malignancy." Such an explanation really reveals nothing and does not seem appropriate to these observations. Willis (*9*) has stated behavioristic criteria for malignancy which are compatible with Ewing's classic definition and fit the circumstances in this work well. The tumors used here have not been observed to differ significantly with respect to their power to infiltrate and destroy adjacent tissues; metastases have not been observed (by inspection in the gross specimen) in backcross hosts in either case, although they have been seen in strain C3H hosts; the growth rate is not significantly different in hosts of C3H maternal ancestry under the two conditions of transplantation; and, although the backcross hosts that have received a graft directly from strain C3H donors, do, on the average, survive somewhat longer, the significance of the difference is doubtful. It would be unwise to say that there is *no* difference in the malignancy of these tumors until experiments designed to settle that aspect of the problem are done, but the difference which is emphasized here does not seem to be properly called an increase in malignancy.

Increased "autonomy" has been sometimes suggested as an explanation for an increased ability to grow in an unfavorable environment. In considering the applicability of the term "autonomy" the word is used in the

usual sense of relative independence of external influences. Although the crucial observation was that the tumor acquired an increased ability to survive in an unfavorable population of hosts, and that statement could be rephrased to say the tumor acquired an enhanced independence of whatever forces the host may bring to bear against the tumor, it does not seem proper to use the term "autonomy" here. It might be argued that, having been conditioned by residence in hybrid, the tumor is less subject to the genetically controlled restrictions upon growth in backcross (to resistant strain) animals and that this constitutes a degree of autonomy. Perhaps there is a modicum of truth in that viewpoint; it would be difficult to disprove. However, several facts stand against a too ready acceptance of that interpretation, or at least indicate that if there is any enhancement of autonomy, it is of limited degree. The tumors do not become generally more transplantable insofar as that can be determined by transplantation into 470 strain C animals and 98 second backcross animals. Furthermore, its growth after conditioning is still far from unrestrained. Under the most favorable circumstances it grows in only 30 percent of backcross recipients; it grows there at a slow rate when compared with that in parent stock or hybrids, and it does not metastasize (as determined by inspection of the gross specimen). It has also been observed that there is apparently a maternal influence at work in these experiments. One aspect of this can be seen in table 1 by comparing the first two lines with the third and fourth lines. In these experiments the maternal influence is unconvincing from a statistical standpoint but because it is supported by previous similar observations (*8*) it appears safe to consider it in this discussion. All this taken together suggests that "autonomy" is not a well selected word in explanation of these findings.

The arguments put forward against increased malignancy or autonomy are for the most part also applicable against "virulence" as conceived by Ehrlich (*11*) and Apolant (*12*) early in the history of cancer research. Although this concept has been frequently used in the past to explain increased powers for growth, it appears that in relation to these experiments the term should be mentioned only to be abandoned.

The questions that arise from the use of the word "mutation" seem to have greater importance in this work and, at the same time, to be more difficult of resolution. If one speaks of the change observed in the tumor used here as a mutation one presumably means a gene change such as is subject to Mendelian inheritance in germ cells and relatively permanent. No means is at hand for determining the first two, more critical, characteristics. The third characteristic will emerge from future work, but it will not give information upon which a decision might rest unless the change turns out to be temporary. In order to determine if the observed change in growth potentialities is permanent or temporary, tumors are being taken from hybrid and backcross donors and returned to strain C3H recipients. After growth in strain C3H hosts for various numbers of generations the tumors are again inoculated into backcross recipients. Such alternating transfers should throw some light on this aspect of the problem.

There is little doubt that Strong, by showing that mutations occur in tumors, has supplied the correct explanation for many of the fortuitously observed spontaneous changes in transplantability that have been reported in the past. However, the circumstances accompanying the spontaneous mutations observed by Strong were quite different from those reported here. The parent stock of the tumor used here has shown no spontaneous change in transplantability and it remains subject to the usual genetic restrictions upon transplantation except for the change reported herein. Furthermore, it should be emphasized that previously reported spontaneous mutations appear to be of a random sort and not repeatable, whereas the change reported herein is of a more specific kind and has been repeated. This same distinction would also apply to mutations induced by physical and chemical means (*13–16*).

If one assumes that during residence of the tumor in a hybrid a mutation has occurred one is confronted with a genetic change which occurs repeatedly, in similar degree and kind under similar circumstances. It appears necessary to call such a mutation an *induced*[3] mutation—one inducible at will by environmental influences. That corollary may seem to be quite enough reason to abandon the term mutation in the interpretation of these results regardless of the outcome of the continuing experiments mentioned, but perhaps it is not. Dobzhansky (*10*), in a recent essay on heredity, environment, and evolution, said,

> "In the last analysis, every mutation is caused by environmental influences and there is no theoretical reason why geneticists could not eventually learn to induce at will specific mutations in specific genes." Quoting further, "The most accurate, although metaphorical, way of describing the dependence between evolution and environment is to say that environment provides the challenges to which organisms may or may not respond by adaptive modifications."

It appears best for the present to leave this interpretation open. Further information may aid in deciding whether the change observed in this work is or is not a mutation.

Adaptation is the word best suited at the moment to describe this change. In part this is due to the fact that this term is a looser one. It is defined as a modification which fits the tumor more perfectly for existence in its environment. This is the sense in which Bashford (*17*) used the word, although he also considered decreased transplantability under certain conditions.

In calling this change an adaptation there is no implication of the mechanism involved. The term as used here means simply that the tumor tissue responds to one environment in such a way as to increase its power to grow in another environment containing the same foreign elements. An attempt to put this into more precise words or to identify the underlying mechanism at this stage would be too speculative. It is in keeping with our working hypothesis to consider that the change is of an

[3] The word "induced" is used here and in the succeeding discussion to indicate that the change is not spontaneous but is produced in response to extrinsic conditions imposed. It is not possible to decide at present whether the ultimate reaction is one of initiation or merely a selective mechanism imposed upon intrinsic attributes of the tissue.

immunologic kind, and it is hoped that experiments may be designed to throw light on this possibility. However, difficulties may be anticipated from the fact that many workers have found that humoral antibodies may not be demonstrable in this sort of thing [Medawar (*18*) and Woglom (*19*)] although Gorer (*20, 21*) has made a start in this direction. It is also pertinent to note Gorer's (*22*) statement that "tumors undergo some antigenic simplification during transplantation."

Whatever the explanation, it is a fact that during growth in F_1 hybrids the tumor underwent some adaptation which enhanced by threefold its ability to survive and grow in backcross animals. The change apparently occurs during the time that the tumor is growing in hybrids; it occurs early, and it does not increase in amount after long residence in hybrids, so far as we can determine. This is sufficient evidence to indicate that there is an interplay of forces between the tumor and its host that may modify the tumor in an important respect. The genetic laws of transplantation cannot explain these results. This is not at all to say that these results disprove the genetic control of transplantation. Such an interpretation is not tenable. The genetic laws are based upon vast data and appear unassailable in the main. However, these experiments using genetics as a basis have shown that other influences are also at work, and neither genetic constitution nor spontaneous mutation is sufficient to explain all the observations of changes in transplantability in tumors.

One important general implication of this work is that if one transplants tumors from one environment to another, either for technical reasons or because of the vicissitudes of animal supply, the assumption that one is dealing with a tumor that is physiologically unchanged may not be justified.

SUMMARY

A tumor which originated in strain C3H and grows in all animals of that strain but will not grow in strain C mice, was inoculated into (C3HC × C) BC mice under two conditions. In one case the tumor was transplanted directly from strain C3H donors to backcross recipients, in the other case the tumor was first transferred from C3H donors to (C3H × C)F_1 hybrids and then, after growth, daughter tumors were transferred to backcross recipients. In the latter case three times as many backcross recipients were killed by a progressively growing tumor as in the former case. The usual genetic laws of transplantation do not explain this result.

The underlying mechanism of this physiologic change was not revealed by these experiments. However, in considering possible explanations of the phenomenon changes in "malignancy," "autonomy," and "virulence" are not believed to be adequate explanations. It is within the realm of possibility that the change represents an induced mutation, but for the moment it is best to describe it more loosely as an induced adaptation in the tumor.

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BLOOD PENTOLYSIS IN RATS BEARING INDUCED SARCOMATA [1]

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The presence of a pentolytic property in the blood of cancer patients has recently been reported by Menkès [2] (*1–4*).

The present study [3] was undertaken to determine whether such a property exists in the blood of experimental rats bearing sarcoma and whether it is a late or an early manifestation of the phenomenon of carcinogenesis of the rats so treated. For this purpose, 57 experimental and 15 control rats of albino stock were employed. The experimental group was injected with a single subcutaneous dose of 20-methylcholanthrene (3 mg. per 0.5 ml. of mineral oil) in the abdomen, the control group being injected in like manner but with mineral oil only. All blood samples were drawn aseptically by intracardiac punctures except for the first 12, which involved decapitation as indicated in table 1.

The minimum quantity of blood required for the pentolytic determination [4] was 0.2 ml.; in general, 0.5 ml. blood samples were drawn into a sterile 2 ml. syringe containing 0.5 ml. of a sterile 5 percent solution of sodium citrate. All citrated blood samples were immediately placed in a cold-room having a temperature of 5° C., and determinations were carried out within 24 hours in all cases except 4 as indicated in table 2. Apart from the 12 decapitated rats, no animals were voluntarily killed. Deaths due to "pneumonia" apparently have no influence on the pentolysis, for autopsy findings of this condition were made for both groups of rats, and the control group remained unchanged (table 3), while the percentage pentolysis for rats of the experimental group increased with time (table 2). The autopsies were performed as soon after death as possible, the animals being kept at 5° C. during any lapse of time. The tissues were fixed in 6 percent formol, embedded in paraffin, cut at 10 micra, and stained with hematoxylin and eosin. Where necessary, serial sections were made.

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[4] To the plasma (obtained by centrifuging citrated blood sample for 10 minutes) was added 0.5 ml. of a 0.1-percent pentose solution. For this purpose d(−) ribose was used, but l(+) arabinose or d(+) xylose can be employed. The mixture was incubated at 37° C. for 2 hours, and the determination carried out by Brachet's (*5*) micromethod with furfural. A Hilger-Spekker colorimeter was used for the readings, and the sugar remaining was read from a calibrated curve. The value of a blank was subtracted, as some blood samples contain pentose. The decrease of the sugar added indicated the *pentolysis*. The determinations involved an error of ±2 percent. All determinations were carried out by Dr. André Bopp.

TABLE 1.—*Percentage pentolysis and day of the final blood sample as compared with the pathologic anatomy and survival time in days of rats of the experimental group*

Survival time in days	Final blood sample		Pathologic anatomy	Rat No.
	Day	Percent pentolysis		
5				21
7				39
7				23
10				19
16				51
32				17
32	32	[1] 0	Reaction [2]	61
34			Reaction	62
34	34	[1] 0	Reaction	18
34			Reaction	41
37			Reaction	56
39	39	[1] 0	Reaction	63
40			Reaction	57
46			Reaction	58
53	53	[1] 0	Reaction	64
58	58	[1] 0	Reaction	65
61	61	[1] 0	Suspect [3]	20
75			Suspect	13
76	76	[1] 0	Reaction	24
79	79	[1] 20	Suspect	25
82	82	[1] 12	Suspect	27
85	85	[1] 4	Suspect	26
92	92	[1, 4] 27	Suspect	14
94			Suspect	9
110	110	[1] 15	Suspect	38
131			Reaction	46
142	104	9	Sarcoma	32
143	130	18	Sarcoma	29
147			Suspect	52
149	130	9	Sarcoma	30
154			Sarcoma	42
158	158	[4] 50	Sarcoma	37
162	162	20	Suspect	44
162	162	25	Sarcoma	45
170			Suspect	53
177			Sarcoma	55
178			Sarcoma	54
188	158	6	Sarcoma	34
197	122	4	Sarcoma	22
198	198	20	Sarcoma	16
205	205	18	Sarcoma	31
208	208	10	Sarcoma	12
210	205	10	Sarcoma	28
212	198	19	Sarcoma	43
212	198	21	Sarcoma	35
212	212	20	Sarcoma	59
216	205	26	Sarcoma	40
219	219	20	Sarcoma	49
244	[5] 198	20	Sarcoma	36
248	[5] 236	10	Sarcoma	50
254	246	[6] 1	Sarcoma	33
281	[5] 236	20	Sarcoma	15
297	285	14	Sarcoma	11
306	285	16	Sarcoma	10
314	[5] 234	26	Reaction	60
322	[5] 236	12	Reaction	47
Living	[4] 270	19		48

[1] Blood samples by decapitation.
[2] Reaction is discussed in the text and is abbreviated as *R.* in table 2 and is *not* the same as that in table 3.
[3] Suspect of sarcomatous changes; see text.
[4] Incubation period of 4 hours.
[5] Utilized for further experiments after this day.
[6] See text and table 2.

RESULTS AND DISCUSSION

The survival time was assumed to be from the day of the injection, all rats being injected within a 2-day interval. Table 1 lists percentage pentolysis and day of the final blood sample and survival time in days as compared with the pathologic anatomy of all rats of the experimental group. Of 57 experimental rats, 25 developed fibrosarcomas at the site of injection, which varied in pattern from spindle-cell type to mixed-cell sarcomas with giant tumor cells (fig. 1). Infiltration of the tissues with free blood (fig. 2) due to the rupture of the primitively constructed vessels and areas of necrosis (fig. 3) were frequent features of these fibrosarcomas in accordance with their malignant nature. No metastases were found.

In the 25 cases in which a sarcoma was histologically identified, 22 had a final blood sample available for comparison of the percentage pentolysis. Of these 22, the final blood sample showed a percentage pentolysis that was positive (5 percent or greater) in 20 cases. The 2 exceptions were rats Nos. 22 and 33 with values of 4 percent and 1 percent, respectively. Rat No. 22 died 75 days after this pentolytic determination, with a tumor weighing 50 gm.; and, from the data in table 2, it appears justified to assume that a later percentage would have been positive. Rat No. 33 showed 3 increasingly positive values for the percentage pentolysis prior to this final pentolytic determination (table 2), and at autopsy had a tumor weighing 125 gm.

Apart from the 20 positive pentolyses found, 7 additional positive values were found for rats Nos. 14, 25, 27, 38, 44, 47, and 60. The last 2 rats, as well as rats Nos. 15, 36, 38, and 50 were utilized for further experiments after the day of the final percentage pentolysis as indicated in tables 1 and 2, and a more extensive examination is being conducted to establish the correlations implied from the preliminary study of these 6 rats for reasons aside from this communication. The first 5 rats showed histological evidence that was suspect of sarcomatous changes, but not clear-cut enough to warrant a diagnosis of sarcoma. Of 6 additional cases that were suspect (rats Nos. 9, 20, 26, 42, 52, and 53), values for percentage pentolysis were available only for rats Nos. 20 and 26. These 2 rats survived 61 and 85 days with a 0 percent and 4 percent pentolysis, respectively, at their time of death. The survival time of rat No. 20 (61 days) initiates the period when a definite diagnosis of sarcoma could not be made, and was the only rat suspect of sarcomatous changes that was definitely negative, taking into account the error of ± 2 percent.

In all rats suspect of sarcomatous changes, an infiltration of the muscle fibers by fusiform cells, situated in a scanty interstitial substance, was observed (fig. 4); these cells had very basophilic nuclei and little cytoplasm. Some of the rats suspect of sarcomatous changes showed a thick perivascular sleeve composed of cells varying in size and shape with very little stroma, there being no sharp limit between the vessel wall and these persivascular cells. These aspects of the reaction to the 20-methylcholanthrene injection were quite different from that seen in the reaction to the injection of the mineral oil only, as seen in the rats of the control group;

TABLE 2.—*Results of pentolytic determinations for rats of the experimental group*

Pentolytic determination												Survival time in days	Path. Anat.	Rat No.
No. 1		No. 2		No. 3		No. 4		No. 5		No. 6				
Day	Percent	Day	Percent	Day	Percent	Day	Percent	Day	Percent	Day	Percent			
[1] 61	0											61	Susp [2]	20
68	0	110	4									197	SA [3]	22
[1] 76	0											76	R [4]	24
[1] 79	20											79	Susp	25
[1] 82	12											82	Susp	27
[1] 85	4											85	Susp	26
88	16	130	18									143	SA	29
88	2	130	9									149	SA	30
90	0	130	3	172	5	205	10					210	SA	28
92	8	130	21	172	22	205	26					216	SA	40
92	[5] 27											92	Susp	14
95	3	135	3	177	[6] 2	205	10					208	SA	12
95	0	135	6	177	[6] 2	219	11	246	9	285	16	306	SA	10
103	1	138	7	178	13	219	16	[7] 236	20			281	SA	15
103	11	138	14	178	[6] 12	205	18					205	SA	31
103	10	138	14	178	[6] 5	219	15	246	15	285	14	297	SA	11
104	9											142	SA	32
109	0	158	6									188	SA	34
109	3	158	8	198	21							212	SA	35
109	8	158	11	[7] 198	20							244	SA	36
109	0	158	5	198	11	215	12	246	[8] 1			254	SA	33
[1] 110	15											110	Susp	38
116	3	157	18	196	19	212	20					212	SA	59
116	0	157	7	196	24	213	26	[7] 234	26			314	R	60
138	6	178	15	198	20							198	SA	16
158	[5] 50											158	SA	37
159	2	198	10	215	12	[7] 236	12					322	R	47
162	20											162	Susp	44
162	25											162	SA	45

162	16	198	19									212	SA	43
215	5	219	6	[7] 236	10							248	SA	50
219	20											219	SA	49
219	13	246	16	[7] 270	19							Living		48

[1] Blood samples by decapitation.
[2] Susp. signifies suspect of sarcomatous changes histologically; see text.
[3] SA signifies fibrosarcoma.
[4] R. signifies reaction seen histologically as a result of the injection of 20-methylcholanthrene in mineral oil when no sarcomatous changes are suspect, and is *not* the same as that in table 3.
[5] Incubation period of 4 hours.
[6] Pentolytic determination after 24 hours.
[7] Utilized for further experiments after this day.
[8] See text.

TABLE 3.—*Results of pentolytic determinations for rats of the control group*

Pentolytic determination												Survival time in days	Path. Anat.	Rat No.
No. 1		No. 2		No. 3		No. 4		No. 5		No. 6				
Day	Percent	Day	Percent	Day	Percent	Day	Percent	Day	Percent	Day	Percent			
83	[1] 10	123	0									124	R[2]	72
90	2	128	1	170	3	213	3	283	3	[3] 316	3	Living		73
93	3	175	1									175	R	74
108	1	157	2									242	R	75
160	1											172	R	77
160	1	283	6	306	4							Living		67
175	1											218	R	76
199	5											201	R	68
244	2	316	2									316	R	80
244	2	[3] 316	2									331	R	69
244	3	[3] 316	3									Living		70
												67	R	71
												239	R	78
												242	R	79
														[4] 66

[1] Blood sample was visibly contaminated.
[2] R. signifies reaction seen histologically as a result of the mineral oil injection, and is *not* the same as that in table 2.
[3] Utilized for further experiments after this day.
[4] Taken out of control group because partially consumed by other rats.

the latter reaction was distinguished by the presence of histiocytes and fibroblasts (fig. 5).

Figure 1 illustrates the cell variability in all the sarcomas induced, and figures 2 and 3 show typical fields of hemorrhage and necrosis in the tumors of the experimental group.

The data in table 2 show the percentage pentolyses increasing with time and are presumably related to the evolution of the induced sarcomas. Five tumors weighed over 100 gm. at autopsy; one completely infiltrated the large intestine with a combined weight of 265 gm. (approximately 40 percent of the total weight of rat No. 10). The wide variation in the weight of individual tumors, the occurrence of necrosis, hemorrhage, and inflammatory reaction in the tumors at time of death, and the insufficient number of determinations of the percentage pentolyses do not as yet permit correlating the size of the tumor and the percentage pentolysis. From work in progress, it appears that the removal of the induced tumor decreases the percentage pentolysis; this is in accordance with clinical results (*2*, *4*). It is interesting to note that a stock albino rat with a spontaneous fibroadenoma of the breast gave negative values of pentolysis on two separate occasions prior to its surgical removal.

The rats of the control group showed a uniform lack of pentolysis for the most part, as indicated in table 3, the only notable instance being rat No. 72 whose blood was visibly contaminated and later gave a negative value.

The use of other carcinogenic agents, animal strains prone to spontaneous tumors, and transplants[6]—all giving similar results—is to be expected from the clinical studies (*2*, *4*). The problem of necrosis is of importance in that the question arises whether there occurs a simple diminution of sarcomatous tissue as a result of degeneration or whether more complex factors are involved—from the point of view of the pentolytic determinations. The possibility of hemorrhage into the tumor with consequent modification of the concentration of the pentolytic factor in the circulating blood cannot be excluded. It is to be expected that the activity of the pentolytic factor would be little affected if there is a low concentration of the pentolytic factor, but might be appreciably affected if there is combination with the products of reaction or an increased substrate concentration.[7]

By doubling the normal incubation period of 2 hours, an approximate doubling of the percentage pentolysis in rats has been found to occur. Upon this basis, a more detailed study of the pentolytic property in the blood of experimental sarcoma-bearing rats during the first 4 months is being undertaken to determine whether the pentolysis is prior to the sarcomatous changes or a manifestation thereof as suggested from the data herein.

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[6] Fourth generation transplants, from a fibrosarcoma induced as described herein, are under observation. Unpublished experiments of S. N. Steen.

[7] The plasma pentose level is increased in the rat after trauma (*6*, *7*).

SUMMARY

Of 57 experimental sarcoma-bearing rats, autopsy findings showed fibrosarcomas at the site of injection for 25 rats of this experimental group. For these 25 rats, 22 blood samples prior to autopsy were available for comparison of the percentage pentolysis; 20 showed a percentage pentolysis that was positive (5 percent or greater). The 2 exceptions and 7 additional positive values are discussed.

Of 10 rats in the experimental group that showed no histological evidence of fibrosarcomas from the 32d to the 58th day, 5 blood samples were available and showed no pentolysis.

Fifteen control rats showed a uniform lack of pentolysis for the most part, there being 1 contaminated sample that gave an initial positive value—later negative.

The conclusion drawn from the results of this 10-month experiment—restricted to experimental sarcoma-bearing rats of albino stock, using 20-methylcholanthrene as the carcinogenic agent—is that a pentolytic property, not normally present in the blood, appears therein as a manifestation of carcinogenesis.

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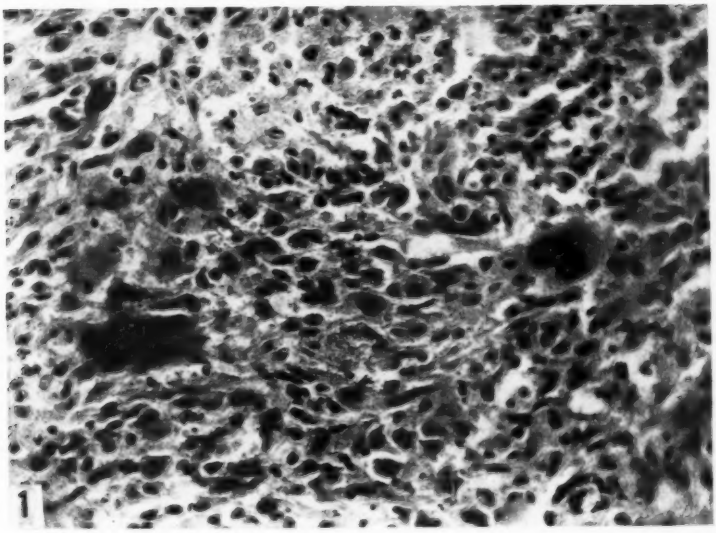
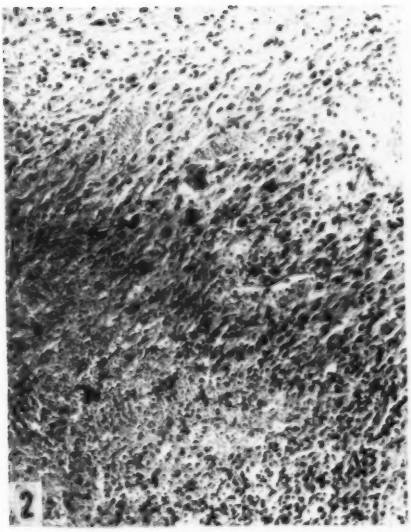
PLATE 2

FIGURE 1.—Fibrosarcoma of rat No. 32 of the experimental group. Tumor weighed 35 gm. at autopsy on the 142d day. Pentolytic value on the 104th day was 9 percent. Note the irregular arrangement of the cells, varying in size, shape, and nuclear configuration, and the giant-cell types. High-power magnification.

FIGURE 2.—Same rat as in figure 1. Note infiltration of the tissues with free blood and primitively constructed vessels. Medium-power magnification.

FIGURE 3.—Same rat as in figure 1. Shows hemorrhage and necrosis. Medium-power magnification.





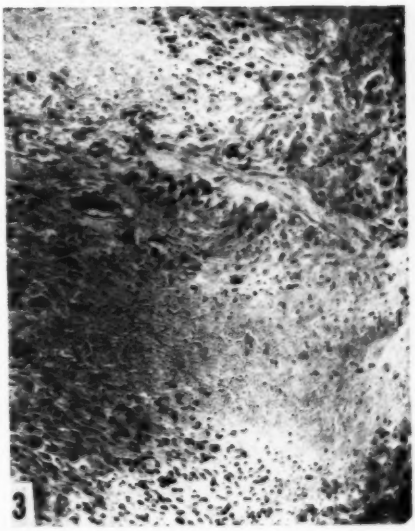


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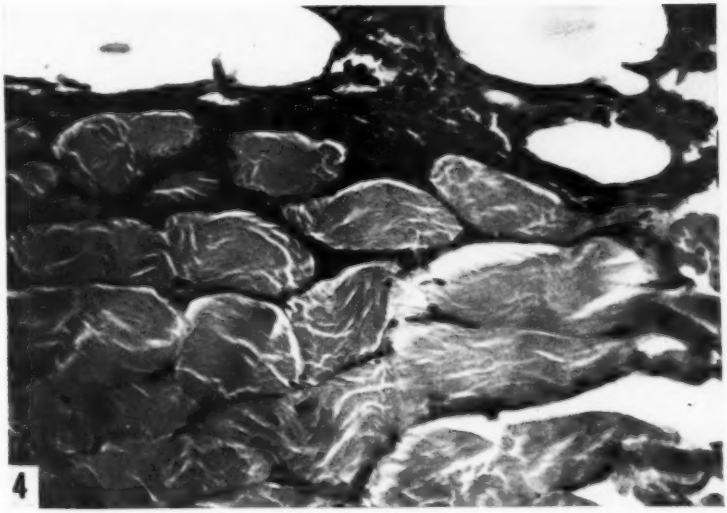
PLATE 3

FIGURE 4.—Rat No. 26 of the experimental group. Suspect of sarcomatous changes. Autopsy and negative pentolysis (4 percent) on the 85th day. Note the infiltration of the muscle fibers by fusiform cells having very basophilic nuclei and little cytoplasm, the scanty interstitial substance, and lacunae. High-power magnification.

FIGURE 5.—Rat No. 77 of the control group. No evidence of sarcomatous changes. Autopsy on the 172d day, and negative pentolysis on the 160th day—after the injection of 0.5 ml. of mineral oil. Note the vacuoles, lymphocytes, fibroblasts, and histiocytes (lower left). High-power magnification.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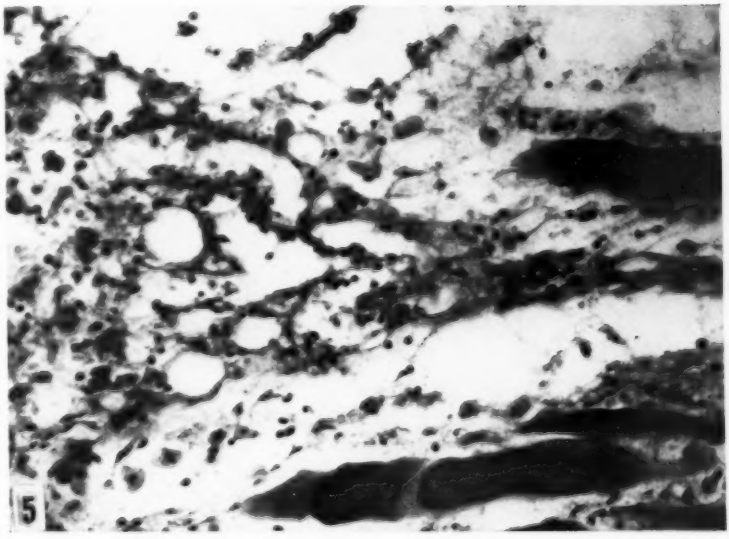
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ATTEMPT TO DETECT A MAMMARY TUMOR-AGENT IN STRAIN C MICE BY ESTROGENIC STIMULATION [1]

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An earlier publication (*1*) recorded a high incidence of mammary tumors in first generation hybrid females born to low-mammary-tumor strain C females (*2*) and high-mammary-tumor strain C3H males (*3*). This finding was contrary to those usually attending similar crossings of such strains because the offspring do not acquire the mammary tumor-agent when suckled by their low-tumor-strain mothers.

It was assumed that if the agent was involved in the occurrence of tumors in the (C × C3H) F_1 hybrids it was transmitted to them by way of strain C milk and was a relatively weak agent because the hybrids developed tumors at a much later average age than either mice of strain C3H or those of strain C that had acquired the strain C3H agent (*3*).

Consequently, a series of experiments was designed to detect, if possible, a mammary tumor-agent in strain C females. The primary objective of these investigations was to expose or unmask a latent virus in a strain of mice, or in their hybrids, which was known to be susceptible to the mammary tumor-agent. Thus far, all such efforts have been unsuccessful. Foster nursing of highly susceptible mice by strain C females did not produce tumors in the fostered animals (*1*), and passage through successive generations of highly susceptible mice did not enhance the activity of an agent, if any, in strain C milk (*2*). Descendants of C females and C3H males, which were genetically more susceptible to the agent than their strain C ancestors, did not show high incidences of mammary tumors (*4*), and exposure of young strain C females to whole-body X-radiation did not activate a latent agent in them (*5*).

This report records briefly an effort to detect a mammary tumor-agent in strain C females by the use of estrogenic stimulation. It was thought that if tumors arise in agent-bearing mice because estrogenic stimulation activates a latent agent, then the same stimulus may activate a weak or masked agent in strain C females, and C × C3H offspring born to and suckled by these females would develop mammary tumors at an early age.

The experiment failed to expose an agent in strain C mice but the occurrence of tumors in their (C × C3H) F_1 hybrid offspring furnished interesting data to compare with results obtained in similar hybrids of other experiments in this series.

[1] Received for publication, April 10, 1950.

MATERIALS AND METHODS

All animals were raised in this laboratory, maintained under similar environmental conditions, and given an unlimited supply of tap water and Purina laboratory chow pellets. While under observation for the appearance of mammary tumors they were kept eight to a cage and were examined every other week for palpable tumors.

Histologic sections were prepared from every tumor by fixing in Tellyesniczky's fluid (20 parts 70 percent alcohol, 2 parts formol, and 1 part acetic acid) and staining with hematoxylin and eosin. The tumors could be divided into four groups according to their histologic appearance as described in an earlier paper (*5*). Three of the groups were adenocarcinomas. One (type A) included the usual or "typical" mammary tumor commonly found in mice harboring the agent. The acinar structure predominated in these tumors. Another (type B) showed little or no evidence of acinar formation but revealed variable patterns sometimes consisting of solid groups of epithelial cells, single layers of cuboidal cells lining cystic spaces or developing as papillary projections into cysts, or cords of cells separated by a heavy stroma. The third (type C) was easily distinguished and consisted of many small cysts lined by a cuboidal epithelium closely invested by a layer of spindle cells. Tumors of the fourth group showed large areas of stratified squamous epithelium, and these were designated as adenoacanthomas. All mammary tumors mentioned in this paper were diagnosed by Dr. Thelma B. Dunn of the National Cancer Institute.

Two procedures were used to test for the presence of the mammary tumor-agent in tumors and tumor-bearing mice. These were as follows: (1) Inoculation of pieces of spleens from tumor-bearing mice into test mice susceptible to the agent. All implants were placed subcutaneously in the right axilla by means of the usual trocar technique. (2) Administration of tumor extracts to susceptible mice. Tumor extracts were prepared by grinding one part by weight of tumor tissue with sand, and diluting in five parts of phosphate buffer (0.1 M, pH 8.0). The mixtures were kept at room temperature for approximately 5 minutes until the heavy particles settled. The supernatant was then used to inoculate the test animals, each mouse receiving 0.2 cc. intraperitoneally.

Test mice for the inoculation of spleen or tumor extracts were 7-to-14-day-old strain C female mice which were genetically resistant to the growth of the implanted tumor tissue. These test animals were bred when 2 months old and permitted to bear one litter, which was removed soon after birth. They were then observed for the appearance of tumors.

Stilbestrol was used to produce estrogenic stimulation. Pellets containing 2, 5, or 10 percent stilbestrol dissolved in cholesterol were prepared according to the technique described by Shimkin and White (*6*). Each pellet weighed approximately 2 mg. and was inserted subcutaneously into the interscapular region. The pellets were removed through an incision in the skin.

EXPERIMENTAL PROCEDURES AND RESULTS

The experiment was started by brother-to-sister matings of 26 females of the strain C colony maintained in this laboratory (*2*) and 92 strain C mice belonging to 26 litters were obtained. When these mice were 10 to 38 days of age half of each litter were given a pellet containing 2, 5, or 10 percent stilbestrol; remaining litter mates served as untreated controls. The pellets were removed 1 month after insertion, and the entire litter was then bred to a strain C3H male.

After supplying hybrids for the experiment, the C females were kept continuously with C3H males until each had produced from four to eight litters. Young born subsequent to the experimental hybrids were killed soon after birth. C females that had carried stilbestrol pellets produced as many litters as their untreated litter mates.

All C × C3H hybrids were fathered by C3H males from an agent-free line of this strain in which only 3 percent of breeding females develop mammary tumors (*7*). Agent-free C3H males were used because it was known that the tissue of the seminal vesicles and the material contained in the lumen from high-tumor strain C3H males contained the mammary tumor-agent (*7*), and when these males were bred to C females a few of their offspring developed mammary tumors at a very early age and harbored the agent (*4*, *5*). Use of agent-free males throughout this experiment should have obviated the possibility that their hybrids would acquire the fathers' agent.

A total of 230 C × C3H hybrids was born to C females and agent-free C3H males. Of these, 156 were born to control C females, 22 to C females that had carried 2 percent stilbestrol pellets, 29 to those that had carried 5 percent stilbestrol pellets, and 23 to those that had carried 10 percent stilbestrol pellets. Because of animal space limitations, only 2 females from litters born to C females that had been exposed to stilbestrol were kept, whereas all the offspring of the control females were kept. This was done because some of the C × C3H hybrids raised by normal C females were given stilbestrol pellets, and others were exposed to X-radiation on the assumption that their genetic constitution may have been more conducive to activation of the agent than that of their C strain mothers. Twenty-three hybrids received a 10 percent stilbestrol pellet when 17 to 31 days of age, and the pellets were removed approximately 1 month later. Forty-two received 100 r of whole-body X-radiation within 24 hours after birth. This was done because the results of another concurrent experiment had, in its early stages, suggested that irradiation of C females may have increased the activity of the agent (*5*).

When the hybrids were 2 months of age they were placed with males of the same derivation and kept with them continuously until most had borne from four to seven litters. All their offspring were removed within a short time after birth. The hybrids were then observed for the appearance of mammary tumors until they were 22 to 25 months of age, when they were killed.

The incidence of mammary tumors is summarized in table 1. Five

TABLE 1.—*Occurrence of mammary tumors in strain C female mice, some of which had received stilbestrol-cholesterol pellets subcutaneously when 10 to 38 days of age and in F_1 hybrid females born to the C females and agent-free strain C3H males*

Group No.	Derivation	Concentration of stilbestrol received in 2 mg. pellet	Concentration of stilbestrol received by C mother in 2 mg. pellet	Number of mice	Mice developing tumors / Mice dying without tumors — Age in months																				Total	Tumor incidence	Average age when tumors arose	Average age at death without tumor
					6	7	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27				
		Percent	*Percent*																							*Percent*	*Months*	*Months*
1	Strain C	0		44		0/1				0/3	0/1	1/0	0/1	0/3	0/6	0/3	0/5	0/3	0/3		0/3	0/5	0/5	1/0	2/42	5	21	20
2	" C	2		15			0/1	0/1				1/0		0/1	0/2	0/2	0/1	0/1	0/2		0/1		0/2		1/14	7	15	20
3	" C	5		18		0/1	0/1			0/3	0/1		0/1		0/3		0/2		0/1			0/1	1/1	0/2	1/17	6	26	18
4	" C	10		15	0/1							0/1			0/2	0/3				1/1	0/1	0/2	0/3		1/14	7	23	21
1A	C × C3H		0	91					0/3		0/1	0/2	2/0	2/1	2/1	0/3	5/1	2/2	3/20	4/12	1/16	0/8			21/70	23	20	22
2A	" " "		2	22					1/0	0/1	1/0	1/0			1/1	1/0	4/1	1/0	0/1	0/1	2/3	0/2			12/10	55	19	22
3A	" " "		5	29						0/1				1/0	0/2	1/1	3/1	1/1	1/3	2/4	0/4	0/3			9/20	31	21	21
4A	" " "		10	23							1/0			0/1		1/1	2/1	1/1	2/2	0/3	0/3	0/4			7/16	30	20	23
1B	" " "	10	0	23										1/1	1/2	1/0	3/1	1/1	1/2	1/3	0/4				9/14	39	20	22
1C [1]	" " "		0	42								1/0		3/2	0/2	3/1	0/3	2/2	0/20	0/3					9/33	21	18	21

[1] This group received 100 r of whole-body X-radiation within 24 hours after birth.

strain C females (two controls and three that had been exposed to estrogenic stimulation) developed tumors. One 15-month-old control mouse had cast six litters and developed a Type A tumor. Pieces of her spleen were tested for the presence of the mammary tumor-agent by inoculation into five test mice that lived to an average age of 15 months without developing tumors. She raised six hybrids, all of which died tumor free at a mean age of 22 months. The second 27-month-old control mouse bore five litters and had a Type C tumor. She raised two hybrids; one developed her tumor when 22 months old and the other died without a tumor when 24 months old.

Each of the three strain C females that had carried stilbestrol pellets had given birth to six or seven litters. One 15-month-old mouse developed a Type B tumor. She raised two hybrids, one of which had a tumor when 21 months of age and the other died tumor free at the age of 25 months. The second 23-month-old C female had an adenoacanthoma. Her two offspring died without tumor when 20 and 25 months old. The third 26-month-old mouse developed two tumors; one Type A and one Type B. She raised one hybrid that developed a tumor at the age of 21 months.

Of 230 F_1 hybrids born to the C females and agent-free C3H males 67, or 29 percent, developed mammary tumors. The incidences in the various groups shown in table 1 ranged from 21 percent in those exposed to X-radiation to 55 percent in those born to C females that had carried a 2 percent stilbestrol pellet.

Tumor-bearing hybrids were not limited to litters born to certain strain C females or C3H males. They represented offspring born to 24 of 44 control C females, to 10 of 15 C females that had carried 2 percent stilbestrol pellets, to 9 of 18 belonging to the 5 percent stilbestrol group, and to 7 of 15 in the 10 percent stilbestrol group. A similar random distribution of mammary tumors in old C $\times$ C3H hybrids was recorded in other experiments of this series (*1*, *4*, *5*).

Analyses were made of the tumors occurring in the hybrids to ascertain if any correlation existed between their histologic appearance and the amount of stilbestrol administered to the C mothers of the hybrids as well as the age of the hybrids in which they arose. The only significant correlation was between the types of tumors and the age of the mice. A total of 69 tumors occurred in the 67 hybrids; 2 mice had 2 tumors. Fifteen were Type A adenocarcinomas, 39 Type B adenocarcinomas, 7 Type C adenocarcinomas, and 8 were adenoacanthomas. A similar predominance of Types A and B tumors was observed in a recent experiment (*5*) in which C females were bred to agent-carrying C3H males.

Twenty-six tumors appeared in mice 12 to 19 months of age and, of these, 10, or 38 percent, were Type A and 12, or 46 percent, were type B. Forty-three arose in 20-to-24-months-old hybrids and of these, 5, or 12 percent, were Type A and 27, or 63 percent, were Type B. Tumors of Type B also predominated in the previous experiment (*5*) but, in contrast to the present results, the incidences of those found in mice up to 19 months of age were

63 percent Type A and 33 percent Type B, and the incidences in mice 20 to 30 months old were 31 percent Type A and 48 percent Type B. The reason for differences in incidence of Type A tumors in the two groups of mice is not known.

Five tumor-bearing hybrids were tested for the presence of the mammary tumor-agent by techniques described previously. Three tumors were used to prepare extracts, and 2 spleens were inoculated into the test animals. One hybrid was 12 months, 2 were 14 months, 1 was 15 months, and 1 was 16 months of age. A total of 39 test mice which were 15 to 18 months old was used, and none has developed a mammary tumor. This finding is in conformity with other tests for the presence of the mammary tumor-agent in other older C × C3H hybrids of this series of investigations (*1*, *4*, *5*).

DISCUSSION

The experiment failed to produce any evidence of the activation of a latent tumor agent in strain C females or in their offspring by estrogenic stimulation but provided further information on the occurrence of mammary tumors in F_1 hybrids procured from low-mammary-tumor strain C females and C3H males.

Table 2 summarizes the results of all experiments concluded in this laboratory in which offspring were derived from strain C females and strain C3H males. Six groups of hybrids are presented according to the presence or absence of the agent in their C3H fathers as well as the number of litters the hybrids bore. Only those hybrids developing tumors at a relatively late age and, presumably, free of the tumor agent are included in the table.

Group 1 were the first hybrids used to test for the presence of the agent in milk of strain C mice. They were of interest because the agent was not found in them, and their incidence of 61 percent mammary tumors was the highest of any group that had cast 3 litters apiece.

Hybrids of Groups 2 and 3 were obtained from C3H males that were carrying the agent or were deficient in it. Six mice of Group 2 developed tumors at an early age and contained the agent: they are not included in the table. The remaining hybrids of this group showed a correlation between the incidence of tumors and the number of litters the mice had borne. Group 3 hybrids were fathered by C3H males deficient in the agent and showed consistent low incidences of tumors despite the fact that some gave birth to 6 or 8 litters. In the report (*4*) of this study attention was directed to this discrepancy by pointing out that of 25 F_1 hybrids fathered by one agent-free C3H male none developed a mammary tumor.

Group 4 hybrids contained five mice that developed mammary tumors when 5 to 7 months of age and carried the agent: these are also omitted from the table. Older hybrids showed a high incidence of tumors and, as in Group 2, there was an interesting correlation between the incidence of tumor and the number of litters produced.

Group 5 mice are presented to show that hybrids fathered by agent-free C3H males and permitted to bear but one litter, exhibit a low incidence of mammary tumors.

TABLE 2.—*Summary of occurrence of mammary tumors in C × C3H F_1 hybrids derived from strain C females and strain C3H males*

Group No.	Reference or experiment No.	Derivation of mice [1]	Mice	Litters born	Mice developed mammary tumor	Tumor incidence	Average age when tumors arose	Mice dying without mammary tumor	Average age at death without tumor
			Number	*Number*	*Number*	*Percent*	*Months*	*Number*	*Months*
1	(*1*)	C × C3H+	75	3	46	61	23	29	24
2 [2]	(*4*)	" " "	16	3	4	25	21	12	24
"	"	" " "	14	4	4	29	23	10	25
"	"	" " "	15	5	10	67	21	5	22
"	"	" " "	14	6	10	71	20	4	25
"	"	" " "	14	7	10	71	23	4	25
"	"	" " "	8	8	8	100	23	0	------
3	"	C × C3H−	6	3	2	33	29	4	24
"	"	" " "	16	4	2	12	25	14	24
"	"	" " "	12	5	2	17	20	10	22
"	"	" " "	13	6	1	8	28	12	25
"	"	" " "	3	7	0	0	------	3	26
"	"	" " "	2	8	0	0	------	2	26
4 [3]	(*5*)	C × C3H+	8	4	3	37	22	5	25
"	"	" " "	29	5	20	69	23	9	24
"	"	" " "	34	6	25	74	24	9	23
"	"	" " "	30	7	24	80	21	6	24
"	"	" " "	22	8	19	86	21	3	19
5 [4]	------	C × C3H−	46	1	4	9	21	42	23
6	1	" " "	4	0	0	------	------	4	20
"	"	" " "	2	1	0	------	------	1	22
"	"	" " "	5	3	1	20	23	4	21
"	"	" " "	26	4	4	15	21	22	22
"	"	" " "	66	5	17	26	19	49	22
"	"	" " "	65	6	20	31	20	45	22
"	"	" " "	50	7	19	38	19	31	21
"	"	" " "	12	8	6	50	21	6	21

[1] A (+) sign after C3H denotes agent-containing males; a (−) sign denotes agent-free males.
[2] 6 other mice developed mammary tumors when 4 to 10 months old and contained the agent.
[3] 5 other mice developed mammary tumors when 5 to 7 months old and contained the agent.
[4] Unpublished data.

The present experiment is represented by Group 6 hybrids. They again demonstrated the influence of breeding upon the tumor incidence and confirmed the findings in Group 3 by revealing consistently lower incidences of mammary tumors in hybrids fathered by agent-free C3H males.

This summary of observations to date with C × C3H hybrids emphasizes the occurrence of mammary tumors in F_1 hybrids derived from low-mammary-tumor strain C females and strain C3H males and also permits three general conclusions. The first and most interesting is the presence of the mammary tumor-agent in a few hybrid offspring of C3H males harboring the tumor agent and its absence in those derived from agent-free C3H males. A total of 289 hybrids was born to C females and agent-carrying C3H males and of these, 11, or 3.8 percent, developed mammary tumors at an early age and were shown to possess the tumor agent. A total of 328 was fathered by agent-free C3H males and none developed a tumor at an early age and none was shown to contain the agent. This again suggests (*4*, *5*) that the agent-carrying hybrids obtained the agent from their C3H fathers.

The second point of interest is the definite influence hormonal stimulation exerted upon the incidence of tumors arising in the older hybrids. Three experiments involving mice from C3H males, with or without the agent, showed that hybrids bearing seven to eight litters developed more tumors than those bearing three to five litters. If these tumors arose in the absence of an agent, the findings showed clearly that hormonal stimulation is of prime importance in the occurrence of such tumors.

The third consistent result is the lower incidence of tumors in hybrids derived from agent-free males. As stated previously (*4*) this difference cannot be attributed to the presence or absence of the agent as yet because hybrids of Group 3 were fathered by agent-free males that were separated from the strain C3H colony by 5 generations of inbreeding, and those of Group 6 by males separated by 12 generations of inbreeding. Thus, the genetic constitutions of the agent-free males may have differed from the other males to such a degree that their hybrid offspring may have been genetically more resistant to the development of tumors. Foulds (*8*) encountered the same problem in F_1 hybrids derived from low-tumor strain C57 black females and high-tumor strain R3 males, with or without the agent, and applied the same criticism to his results. This problem is open to investigation and is receiving consideration.

Foulds also pointed out that his tumor-bearing mice were concentrated in particular families, and, as in the few agent-containing hybrids of Groups 2 and 4 of table 2, probably acquired the agent from their fathers. His hybrids differed from the C × C3H hybrids in that the older animals showed a much lower incidence of presumably agent-free tumors.

In this laboratory the presence of the agent in a few young tumor-bearing hybrids and its absence in the older tumor-bearing hybrids (even litter mates of the younger mice) suggested the possibility that the agent was involved in the production of all the tumors. An attenuated or latent agent present in strain C milk could be responsible for the appearance of tumors in old hybrids, and this agent, under certain conditions, could become activated or mutate and incite tumors at an extremely early age in a few mice. Experiments designed to explore this possibility have, thus far, yielded results that indicate the presence of two unrelated problems. One concerns the development of mammary tumors in old mice because of their genetic constitution and intensive hormonal stimulation and the other the sudden appearance of the mammary tumor-agent in a few younger animals of the same hybrid derivation. Up to the present time, experimental evidence suggests that the latter mice acquired the agent from their strain C3H fathers.

The importance of the genetic makeup of hybrid mice in relation to the development of mammary tumors in the absence of the agent was shown previously (*7*) by the hybridization of various strains, all of which were presumably free of the agent but differed in their degrees of susceptibility to the development of mammary tumors when the agent was present. Hybrids born to such strains were permitted to bear only three litters apiece, and a relatively small number developed mammary tumors.

However, the data showed that most of the tumors appeared in hybrid descendants of strains revealing a high incidence of mammary tumors when exposed to the mammary tumor-agent. Thus, there was a parallelism between an inherited tendency to mammary-tumor development and susceptibility to the mammary tumor-agent.

The experiment recorded herein, together with the summary of preceding investigations, extends this observation by demonstrating the importance of hormonal stimulation in the development of mammary tumors in those mice that are genetically predisposed to this tumor but lack the agent.

SUMMARY

An experiment was performed to ascertain whether low-mammary-tumor strain C females harbored a latent mammary tumor-agent.

The C females were exposed to stilbestrol-cholesterol pellets containing 2, 5, or 10 percent stilbestrol when from 10 to 38 days of age; the pellets were removed 1 month later and the mice then bred to agent-free strain C3H males. The C females and their C × C3H hybrid offspring were observed for the occurrence of mammary tumors.

The results failed to reveal the presence of the mammary tumor-agent in strain C females but confirmed the occurrence of mammary tumors in F_1 hybrids born to strain C females and agent-free strain C3H males. In contrast to the results of previous experiments in which the agent was present in a few similar hybrids born to C females and agent-containing C3H males, none of the hybrids fathered by the agent-free C3H males carried the agent. The incidence of tumors in hybrids derived from agent-free C3H males was lower than the incidence in hybrids derived from agent-containing males. Both groups of hybrids, however, showed a correlation between the incidence of tumors and the number of litters produced.

The results are discussed in respect to the roles of the mammary tumor-agent, genetic susceptibility, and hormonal stimulation in the production of mammary tumors in hybrid mice.

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INFLUENCE OF THYMECTOMY, SPLENECTOMY, AND GONADECTOMY ON INCIDENCE OF RADIATION-INDUCED LYMPHOID TUMORS IN STRAIN C57 BLACK MICE [1] [2]

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INTRODUCTION

Previous studies have demonstrated that young male and female mice of strain C57 black are highly susceptible to radiation-induced lymphoid tumors, which almost always originate in the thymus gland (*1*, *2*, *3*, *4*). Furth and his co-workers (*5*, *6*) and others (*7*, *8*) have demonstrated that thymectomy and gonadectomy may profoundly modify the incidence of spontaneous lymphomas in certain strains. The present work was undertaken to determine whether these procedures would similarly affect the incidence of radiation-induced lymphomas.

MATERIAL AND METHODS

The influence of thymectomy and gonadectomy on radiation-induced lymphoma incidence was studied in two separate experiments. Inbred strain C57 black mice were used throughout. In Experiment I, littermate animals were thymectomized, splenectomized, or kept as intact controls and treated with fractionated whole-body roentgen rays at 1 to 2 months of age. A total of 750r was delivered in 10 equal treatments (five daily treatments in two consecutive weeks).[3] Throughout the subsequent observation period, animals were identically caged and maintained on Purina laboratory chow and water *ad libitum*. Animals that died following operation or irradiation, or suffered premature or accidental death during the experimental period, are excluded from the experimental results. Routine autopsies were performed on all other animals, and multiple organs taken for histologic examination.

In Experiment II, mice of both sexes were gonadectomized and littermates kept as intact controls. All were exposed to fractional whole-body roentgen rays starting at 1 month of age. A total of 800r was given in 4 equal treatments at 2-week intervals.[3] Subsequent maintenance, observation, and autopsy studies were carried out as described above.

The technique of thymectomy was somewhat modified from that previously described (*6*, *9*) and will therefore be described in some detail. A combination of light nembutal and light ether anesthesia has been used;

[1] Submitted for publication, April 10, 1950.

[2] These experiments were initiated in the Biophysics Section, National Cancer Institute, Bethesda, Maryland, and have been carried to completion in the Department of Radiology, Stanford University School of Medicine, with the aid of a grant from the National Cancer Institute, U. S. Public Health Service, Federal Security Agency.

[3] Physical factors were: 186 KVP, 20 ma., 0.25 mm. Cu and 0.55 mm. Al filter, focus skin distance 47 cm., output 66r/min.

full anesthetic doses of nembutal result in depression of the respiratory effort while ether alone produces excessive mucous secretion and bronchial obstruction.

A midline cutaneous incision is made starting at the mandible and extending to the lower end of the sternum. The animal is placed on its back with the head toward the operator. Rubber bands are used to strap down the hind legs and tail and another band placed transversely across the open mouth behind the teeth keeps the head extended. The strap muscles and salivary glands are separated by blunt dissection along the midline raphe to the thoracic inlet and the trachea is clearly exposed. After additional brief etherization, a midline incision is made down the sternum with a sharp fine-point scissors starting at the manubrium and extending about 12 to 14 mm. Care must be taken not to injure the underlying heart or great vessels. The skin edges are then grasped with forceps and brought together over the chest to prevent the aspiration of air into the thoracic cavity. The animal will promptly return to a good respiratory state, and the chest may then be opened again.

The thymic lobes will be visualized lying on either side of the midline in the upper mediastinum partially covering the trachea and great vessels. They are extremely soft and friable, and some practice is needed in learning how to grasp them. Small, curved forceps with finely serrated non-toothed jaws are used. With the point of the forceps the caudad end of one lobe is teased loose and gradually lifted free of its fine attachments and stripped toward the head. The firmest attachments occur at the upper end and care must be exercised while stripping the gland to exert a steady pull to avoid tearing the great vessels. When properly performed, the dissection is quite clean and leaves no significant residue. The other lobe is similarly teased free and stripped away. Finally, the upper mediastinum is inspected for residual thymic fragments.

At any point during this procedure the animal may develop profound respiratory distress as a result of the unilateral or bilateral pneumothorax which usually occurs. Dissection of the thymic lobes must then be abandoned abruptly and measures instituted to bring the animal back to good condition. The skin edges of the incision are quickly brought together over the chest to form an air seal. Gentle intermittent pressure on the upper abdomen is then made to bubble air from the thoracic cavity through the skin edges, permitting re-expansion of the lungs. If this is successfully accomplished, the animal will begin to breathe rapidly and easily again. Resuscitation may be required two or three times during the course of the operation.

As soon as thymic removal has been completed the skin edges are brought together and closed with a continuous suture of fine silk starting at the caudal end of the incision. It is not necessary to suture the sternum or muscle layers. Before the final few stitches are placed, it is advisable to bubble residual air out of the chest again by repeated gentle pressure on the abdomen. If the anesthesia is kept very light, and if residual pneumothorax is not excessive, the animal will be partially awake and active at

the completion of the operation. Initial mortality is invariably high, but with practice can be reduced to less than 10 percent. The procedure is easier in young mice because the fibrous attachments of the thymus gland are more delicate and the organ can be stripped away with less risk of tearing the great vessels. Observations at intervals after the procedure have not given evidence of re-growth of the thymus if initial operation is complete.

OBSERVATION AND RESULTS

The results of Experiment I are summarized in table 1. Only 1 lymphoid tumor (4 percent) occurred in the thymectomized group. This tumor appeared at 440 days after irradiation; the mean survival time for the entire group was 392 days after the start of irradiation. In contrast, 48 and 57 percent, respectively, of the mice in the intact and splenectomized groups developed lymphomas after mean intervals of 197 and 184 days following the start of irradiation.

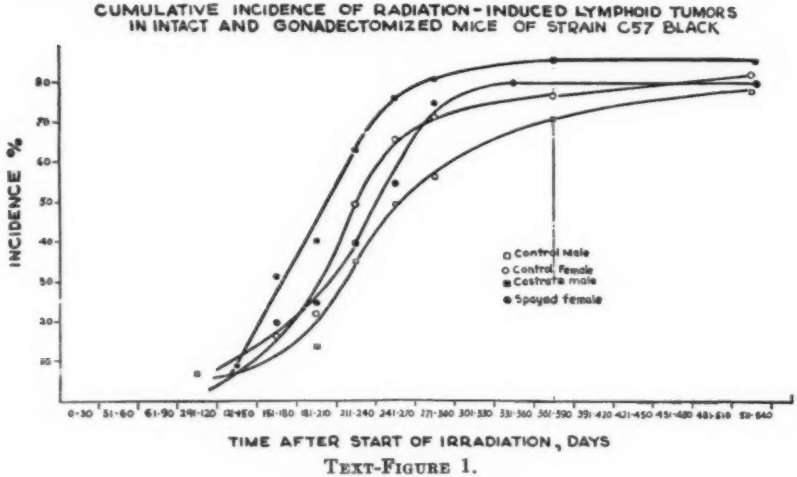
TABLE 1.—*Influence of thymectomy and splenectomy on incidence of radiation-induced lymphomas in strain C57 black mice*

Group	Initial number of mice	Net number of mice	Number of mice with lymphomas	Incidence, percent	Survival time, net number mice	Survival time, mice with lymphomas
					Days	*Days*
Intact irradiated	24	21	10	*48*	319 (134–536)	*197* (134–317)
Splenectomized irradiated	25	23	13	*57*	276 (129–470)	*184* (129–470)
Thymectomized irradiated	41	23	1	*4*	392 (162–457)	*440*

It is difficult to evaluate the origin of the single lymphoma which appeared in the thymectomized group. It must be recalled that strain C57 black mice are not entirely refractory to the spontaneous development of lymphomas. An incidence varying from zero to about 8 percent has been observed; almost all of the spontaneous tumors appear after 400 days of age. It is, therefore, quite possible that the lone tumor observed in the thymectomy group was in actuality a spontaneous rather than a radiation-induced lymphoma. This lesion was morphologically indistinguishable from others in the experiment and from those previously described (*10*). It is concluded that thymectomy prior to irradiation effectively prevents the development of radiation-induced lymphoid tumors in mice of strain C57 black.

Cumulative incidence curves of Experiment II are presented in text-figure 1 and results summarized in table 2. The final lymphoma incidence in all groups was unusually high (79 to 86 percent) and did not differ significantly among groups. Although the mean induction time among

castrate males is somewhat less than that of other groups and considerably less than that of control males, this difference is not statistically significant at the 0.05 level. Analysis at an earlier point in the curve (300 days after the start of irradiation) revealed an incidence of 81 percent among castrate males and 57 percent among control males, but the difference of 24 percent is again not statistically significant at the 0.05 level. Under the conditions of this experiment, therefore, gonadectomy did not appear to influence the rate or frequency of development of radiation-induced lymphoid tumors.



TEXT-FIGURE 1.

DISCUSSION

Furth and his co-workers (*5*, *6*) have previously demonstrated that thymectomy sharply reduces the incidence of *spontaneous* lymphomas and prolongs life in certain susceptible strains. The results of Experiment I indicate similarly that the development of lymphoid tumors following whole-body irradiation is dependent upon the presence of the thymus gland at the time of exposure. It would be interesting to determine whether thymectomy performed at varying intervals following irradiation would also be effective in preventing lymphoma development.

The possible role of the thymus gland in the leukemogenic process has been discussed by Furth and Boon (*11*). They concluded that there was no good evidence that removal of the thymus gland had any systemic effect, and suggested that the effect of thymectomy was most probably due to the removal of an important source of malignant lymphoid cells. This view is supported by the observation that radiation-induced lymphoid tumors of strain C57 black mice originate regularly in the thymus gland and can be demonstrated in this structure before appreciable spread to other tissues has occurred (*3*). There is no similar predilection for the spleen, and both the present experiments and those of Furth indicate that splenectomy does not modify lymphoma incidence.

TABLE 2.—*Influence of gonadectomy on incidence and induction time of radiation-induced lymphomas in strain C57 black mice*

Group	Initial number of mice	Net number of mice	Number of mice with lymphomas	Incidence, percent	Survival time, mice with lymphomas
					Days
Control male	19	14	11	*79*	262 (120–512)
Control female	21	18	15	*83*	244 (153–532)
Castrate male	29	22	19	*86*	215 (146–383)
Spayed female	24	20	16	*80*	234 (155–331)

Despite the pronounced susceptibility of the thymus gland and the distinct inhibitory influence of its removal upon lymphoma development, irradiation localized to the region of this structure does not result in the development of lymphoid tumors in strain C57 black mice (*12*). It is clear, therefore, that some systemic or indirect mechanism must be involved in the induction process, either alone or in combination with a direct effect of radiation on thymic tissue.

In previous studies, gonadectomy has consistently modified the incidence of *spontaneous* lymphomas but the direction of this effect has not always been the same. In the strains studied, susceptibility was influenced by sex, females being more susceptible than males. Furth and his associates (*6*), working with the Ak stock of mice, found that ovariectomy significantly reduced the incidence while orchiectomy yielded an insignificant increase in incidence of spontaneous lymphomas. In contrast, Murphy (*7*) reported that orchiectomy in a Rockefeller Institute strain of mice resulted in an increase in incidence among males while ovariectomy was without effect. The combination of ovariectomy and treatment with testosterone produced a decrease in incidence among females. Using strain C58 mice, Law (*8*) also found an increase in spontaneous lymphoma incidence following orchiectomy but no effect after ovariectomy. The situation is further complicated by the fact that estrogen administration has been shown to be leukemogenic for certain strains of mice (*13*), and that its effect may be related to age (*14*).

The results of Experiment II do not contribute to the resolution of these discrepancies. Gonadectomy does not appear to have influenced significantly either the rate or frequency of development of radiation-induced lymphomas in strain C57 black mice. This is consistent with the previous observation that there is no difference in susceptibility among males and females of this strain when irradiation is carried out at 1 month of age, as in the present experiment. However, previous data (*2*) suggest that a relatively greater decrease in susceptibility occurs in males when irradiation is deferred until 2 months of age or later. It

is possible, therefore, that the discrepant results with regard to the influence of orchiectomy are related to the age at which this operation was performed in relation to the time at which the spontaneous or exogenous stimulus was applied. The use of an exogenous stimulus such as radiation permits determination of precise time relationships and perhaps the role of the male gonads may be clarified by further studies in postpubertal mice.

The possibility that the ovaries exert an influence on leukemogenesis is not excluded by the present experiments. It must be remembered that whole-body irradiation results in sterility and presumably in a decrease in estrogen secretion. It is possible, therefore, that surgical ovariectomy adds little to the effect produced by irradiation alone. The converse experiment of shielding the ovaries from irradiation will be necessary to determine whether or not the ovaries play a part in the genesis of radiation-induced lymphomas.

Comment may be made about the unusually high incidence of lymphomas observed in Experiment II. Irradiation was carried out somewhat differently in this experiment from what has previously been the case. Heretofore, some 10 to 12 daily treatments were usually given in a 2-week period to a total whole-body dose of about 750 to 1000r. In Experiment II, 4 treatments were given at 2-week intervals for a total of 800r. It is possible that the periodicity factor may have been responsible for the augmented response noted. This possibility is being studied in a large scale dose-response experiment now in progress.

Analysis of the cumulative incidence curves in Experiment II (text-figure 1) and those in a previous publication (*2*) reveals that virtually all of the lymphoid tumors induced by irradiation of strain C57 black mice make their appearance within 10 to 12 months after the start of treatment. The sporadic tumors which appear later may well be spontaneous in origin. It would therefore seem desirable and valid in future investigations with this strain to terminate experiments at 300–360 days after irradiation. This will materially increase the rate at which successive variables may be subjected to experimental study.

SUMMARY AND CONCLUSION

Mice of strain C57 black were thymectomized, splenectomized, or kept as intact controls and treated with fractionated whole-body roentgen rays at 1–2 months of age. The technique of thymectomy is described in detail. Of 23 thymectomized mice, 1 (4 percent) developed a lymphoid tumor at 440 days after irradiation. Tumors appeared in 13 (57 percent) of 23 splenectomized and 10 (48 percent) of 21 control irradiated mice at mean intervals of 184 and 197 days, respectively, after irradiation. It is concluded that thymectomy effectively prevents the development of radiation-induced lymphomas. This result confirms the previous observations of Furth and associates, working with spontaneous lymphomas in susceptible strains. Since earlier studies indicated that the thymus

gland is the site of origin for virtually all radiation-induced lymphomas in strain C57 black mice, the inhibitory effect of thymectomy may be attributed simply to removal of this susceptible focus.

In another experiment with mice of the same strain, animals were gonadectomized and litter-mates kept as intact controls. All were exposed to fractionated whole-body roentgen rays at 1 month of age. Lymphoid tumors occurred in 11 of 14 intact males (79 percent), 15 of 18 intact females (83 percent), 19 of 22 castrate males (86 percent), and 16 of 20 spayed females (80 percent). The mean induction times were, respectively, 262, 244, 215, and 234 days after the start of irradiation. These differences in incidence and mean induction time are not significant at the 0.05 level.

Other investigators working with spontaneous lymphomas in various strains have observed striking modifications in incidence after gonadectomy. In some instances, ovariectomy has decreased incidence; in others ovariectomy has been without effect while orchiectomy has increased incidence. The present experiments do not give evidence of either effect on the incidence of radiation-induced lymphoid tumors in strain C57 black mice. Some of the experimental factors which may be related to these conflicting results are discussed.

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DISTRIBUTION OF RADIOACTIVITY IN TISSUES OF NORMAL AND TUMOR-BEARING MICE FOLLOWING INTRAVENOUS ADMINISTRATION OF RADIOIODOTETRAZOLIUM SALT [1]

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Straus *et al.* (*1*) showed that neoplastic tissue of animals *in vitro* reduces triphenyl tetrazolium chloride to a colored formazan more rapidly than does normal tissue. It was postulated that this differential reduction of the tetrazolium salt was due to enzymatic differences between normal and neoplastic tissue, probably the enzymes in the chain of glycolytic fermentation.

To determine the fate of tetrazolium salts *in vivo* Seligman *et al.* (*2*) prepared 2,5-diphenyl-3-*p*-radioiodophenyltetrazolium chloride and reported the distribution of activity in eight mice bearing Walker Sarcoma 37 and 8 normal mice of Swiss strain. Sarcoma 37 was found to contain less radioactivity than most other tissues. Since evidence for purity of the material at hand was available, in contrast to that of Seligman *et al.*, as explained in the Experimental Section, it was considered desirable to compare the distribution of our preparation with the previously published results.

MATERIALS AND METHODS

Preparation of 2,5-diphenyl-3-p-radioiodophenyltetrazolium chloride

The principal aspects of this synthesis already have been described in the literature (*3*, *4*). The details of the oxidation of formazan are presented here, since some of these points may be critical in the obtaining of pure radioactive tetrazolium chloride.

One hundred twenty-two milligrams of N-(*p*-radioiodophenyl)-*N'*, C-diphenylformazan were dissolved in a mixture of 2.4 ml. of chloroform and 2.4 ml. of absolute methanol. After 0.31 ml. of concentrated hydrochloric acid and 0.45 ml. of freshly distilled butyl nitrite were added consecutively, the solution was allowed to stand for 90 minutes. The solution was boiled down to approximately 1 ml. in a flask over a hot plate. Dioxane, 3 ml., was added, and the mixture was again concentrated to incipient crystallization. The mixture was centrifuged, and the solid

[1] Received for publication, April 26, 1950.

[2] This work was done during the tenure of an Atomic Energy Commission Postdoctoral Fellowship in the Medical Sciences of the National Research Council.

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impurity was discarded. To the supernatant was added 3 ml. of dioxane, and the mixture was concentrated to crystallization. After centrifugation, the precipitate was washed with two 1.0-ml. portions of ether and then dried in a vacuum desiccator. The yield was 33 mg. of m. p. 229° C. (dec., corr.).

The m. p. observed approximates that of 232°–233° C. (dec.) found for an analytically pure sample obtained during preparation of a series of tetrazolium chlorides (*3*). The material of Seligman *et al.*, was reported as sintering at 160° and decomposing at 170°. Seligman's compound was not analyzed, however, and no m. p. was reported for a nonradioactive sample which gave correct C–H values. The data presented were thus insufficient for a judgment of purity of Seligman's radioactive tetrazolium chloride.[3]

Distribution of radioactivity in mice

The radioiodotetrazolium salt was made up in physiological saline so that 0.1 ml. contained 0.1 mg. of the salt. In preparing the solution it was noted that a fine sediment developed after standing. The tetrazolium was administered to the animals immediately after it was put into solution.

Eleven strain A mice bearing a transplantable lymphoma, 11 normal strain A mice, 10 strain C3H mice bearing a transplantable mammary adenocarcinoma, and 4 strain C3H controls were used. The lymphomas were used 3 weeks following transplantation, and the mammary tumors were about 1.5 weeks old. Each mouse received 0.1 ml. of the solution by intravenous administration into the lateral tail vein.

At the time intervals indicated in the tables, mice were killed with ether, dissected, and the tissues placed in metal planchets (brass bottle caps) for determination of radioactivity. The wet weight of the tissues was determined, using a torsion balance, and the tissues were counted, using a thin mica end window Geiger-Müller tube (approximately 1.9 mg./cm.^{2}) and a "64" Tracerlab Scaler. An aliquot of the dose was simultaneously counted and the radioactivity of the tissues expressed as percent of the dose per-gram-wet weight of tissue. The counts were corrected for self-absorption by use of "infinite thickness" correction, and for background. The accuracy attained in counting such samples is limited, but is sufficient to detect significant differences. An estimation of the accuracy attained can be surmised from the total recoveries obtained in some of the animals. This ran from 80 to 105 percent.

RESULTS AND DISCUSSION

As given in tables 1 and 2, the present findings are essentially in agreement with those reported by Seligman (*2*). The activity disappeared rapidly from the blood, and about 50 percent was excreted in 24 hours. Both the lymphoma and mammary carcinoma had less radioactivity than most of the other tissues.

[3] Debye-Scherrer photographs of the material used in this study gave a good pattern indicative of the crystalline state; whereas a sample of tetrazolium used by Seligman gave little or no pattern.

TABLE 1.—*Distribution of radioactivity in normal and tumor-bearing (mammary) strain C3H mice after intravenous administration of 0.1 milligram diphenyl-p-radioiodophenyltetrazolium chloride*

[Values given as percent of dose per-gram-wet weight]

	Normal strain C3H mice—hours after injection				Strain C3H mice with mammary carcinoma—hours after injection									
	24		47		1.5			6.0		25			48	
Weight animal, gm	25.1	34.4	35.0	30.3	31.6	30.3	32.5	33.2	31.2	26.7	30.8	32.1	32.1	28.5
Weight tumor, gm	------	------	------	------	.2	.3	.7	.2	.4	.5	.3	.7	.5	1.7
Tumor	------	------	------	------	*2.9	*3.7	*3.5	*2.6	1.8	*1.0	*.6	*2.2	*.8	*.4
Liver	3.9	2.9	.3	.9	13.0	16.6	18.0	4.8	3.5	5.2	3.9	3.9	1.5	1.5
Kidney	4.8	3.5	*.6	1.3	61.0	69.0	76.4	17.8	18.8	10.1	12.5	9.6	*2.6	*4.1
Blood [1]	*.7	*.4	------	------	*5.0	*2.5	6.0	*2.4	*1.2	*.8	*.6	*.6	------	------
Excreta	63.3	52.7	------	------	.8	.5	.5	12.1	------	50.0	50.0	------	------	------
Thyroid gland [2]	*.1	1.1	*.6	*.5	*.1	*1.2	*1.8	1.1	2.5	*.9	.5	*1.0	*.6	*.9
Intestine [4]	3.8	3.1	1.3	1.4	------	------	------	------	------	3.3	2.3	2.6	.9	1.9
Spleen	*.4	*.6	------	------	------	------	------	------	------	*.9	*.8	*1.1	------	------
Muscle [3]	*.7	*.6	------	------	------	------	------	------	------	*1.5	*.4	*.8	------	------
Skin	1.8	1.1	1.0	1.3	------	------	------	------	------	2.2	.9	*.9	2.0	*.8
Lung	*.3	*.5	*.3	*.4	*5.9	7.6	8.9	1.7	1.8	*.7	*.5	*1.2	------	------
Recovery	91.0	86.0	------	------	------	------	------	------	------	96.0	82.0	------	------	------

*Less than 5X background.
[1] Total blood was estimated as 7 percent of body weight.
[2] Percent of dose per whole organ (thyroid).
[3] Total muscle was estimated as 45 percent of body weight.
[4] Intestine and excreta.

TABLE 2.—*Distribution of radioactivity in normal and tumor-bearing strain A mice after intravenous administration of 0.1 milligram diphenyl p-radioiodophenyltetrazolium chloride*

[Values given as percent of dose per-gram-wet weight]

	Normal strain A mice—hours after injection											
	2.0			6–7		24			47			
Weight animal, gm.	24. 7	19. 8	19. 8	20. 7	18. 8	17. 7	17. 1	19. 2	31. 5	22. 7	18. 8	
Weight tumor, gm.												
Tumor												
Liver	32. 6	30. 7	35. 7	7. 5	8. 3	8. 0	4. 1	4. 2	. 6	1. 0	1. 0	
Kidney	77. 0	78. 1	75. 6	18. 7	15. 0	8. 6	5. 1	5. 9	. 9	1. 8	*1. 7	
Blood [1]	9. 4	9. 6	*4. 8	*1. 3	*. 8	*1. 0	*1. 0	*. 8				
Excreta	. 9	. 9	. 9			53. 0	53. 0	53. 0				
Thyroid gland [2]	*1. 9	2. 1	*1. 0	. 7	. 8	2. 2	2. 6	1. 3	. 8	*. 5	*. 2	
Intestine [4]						4. 5	5. 1	4. 8	1. 2	1. 6	2. 1	
Spleen						*2. 3		*3. 6				
Muscle [3]						*2. 9		*1. 9				
Skin						2. 3		2. 7	1. 2	2. 2	2. 9	
Lung	12. 5	13. 7	15. 3	2. 3	2. 2	2. 4	1. 7	*. 9		*. 5	*. 5	
Recovery						102. 0		96. 0				

	Strain A mice with lymphoma—hours after injection										
	1.5–2.0			6–7		23–24			48		
Weight animal, gm.	40. 4	47. 6	32. 4	35. 2	38. 1	41. 2	34. 4	43. 0	37. 6	38. 8	30. 8
Weight tumor, gm.	5. 3	7. 8	4. 7	1. 9	7. 0	9. 6	4. 4	6. 3	5. 0	5. 9	3. 4
Tumor	1. 9	1. 5	1. 4	1. 0	1. 1	1. 4	1. 0	1. 3	. 5	. 5	. 3
Liver	17. 0	14. 9	15. 7	5. 0	8. 0	3. 3	4. 0	3. 3	2. 2	1. 7	2. 6
Kidney	85. 0	87. 7	61. 0	16. 9	15. 5	17. 6	16. 6	18. 0	4. 4	5. 2	3. 9
Blood [1]	6. 2	*8. 3	11. 5	*. 8	*1. 1	*2. 6	*1. 2	*2. 2			
Excreta	. 4		. 4			41. 6	49. 5	41. 6			
Thyroid gland [2]	*1. 6	*2. 4	*1. 4	. 7	. 3	*. 4	*. 3	*. 4	*. 7	*1. 4	*. 6
Intestine [4]									1. 0	. 9	1. 5
Spleen		*8. 0									
Muscle [3]											
Skin										2. 3	2. 9
Lung	9. 6	7. 8	9. 7	1. 3	1. 5	*2. 0	*2. 2	*2. 3			
Recovery											

*Less than 5X background.
[1] Total blood was estimated as 7 percent of body weight.
[2] Percent of dose per whole organ (thyroid).
[3] Total muscle was estimated as 45 percent of body weight.
[4] Intestine and excreta.

The results indicate radioactivity found in the tissues, and represent the distribution of tetrazolium salt only if the radioiodine remains in the tetrazolium salt under *in vivo* conditions. The concentration of radioiodine in the thyroid gland is a check on the *in vivo* ionic exchange of iodine if there is no carrier iodine in the preparation. With carrier present (about 25 to 30 micrograms of iodine per animal in the tetrazolium salt)

the thyroid gland would probably be saturated with inert iodine so that concentration of radioiodine in the gland would be prevented (*4*).

The data were compared with published findings on distributions of activity with tracer and carrier iodine in order to determine in what form the radioactivity was present in the tissues. Pressman (*5*) found that at 24 hours after injection of carrier-free iodine, the kidney and liver of mice contain less than 0.5 percent of the injected radioiodine per gram; whereas the thyroid gland contains about 30 percent of the administered radioiodine. When radioiodine is administered with about 14 mg. of carrier iodine, the thyroid gland in 24 hours contains less than 0.01 percent of the radioiodine, and the kidney and liver less than 0.1 percent per gram. When about 480 micrograms of carrier iodine is administered with the radioiodine in mice, Seligman (*6*) found that more than 33 percent of the activity is excreted in 24 hours and the liver and kidney do not retain any of the radioiodine. The thyroid gland loses all of its radioiodine after 6 hours. Essentially the same findings have been reported for the rat by Perlman, Morton, and Chaikoff (*7*).

The radioiodine in the tissues following injection of radioiodotetrazolium could occur in three possible forms: the tetrazolium or formazan, ionic iodine, or other iodine-containing conversion products of the tetrazolium. The fact that there was about 20 times as much activity in the liver and kidney at the end of 24 hours as compared with the amount of activity present after a tracer dose of radioiodine suggests that most of the activity in these organs is not in the form of ionic iodine. Whether the activity in the liver or kidney is in the form of tetrazolium, formazan, or in iodine-containing catabolic products, cannot be determined from the present data.

Seligman *et al.* (*2*) suggested that the high concentration of radioactivity in kidney and liver following administration of radioiodotetrazolium may be related to the high dehydrogenase activity in these tissues, which consequently may result in more extensive reduction and deposition of formazan in these tissues. It is to be noted, however, that the rate of loss from the kidney and liver is rapid, so that at the end of 48 hours the concentration of radioactivity in these tissues is about the same as in most other tissues studied, (thyroid gland, intestine, lung, and skin). This suggests that the high activity in the liver and kidney is probably due to the excretory detoxifying role of these organs rather than to the deposition of the insoluble formazan salt. If the concentration of radioactivity in these tissues is due to the deposition of the insoluble formazan, the high concentration should be sustained for longer periods, unless after formation of formazan these organs are active in further degrading the formazan to iodine-containing conversion products, which are in turn excreted.

The effect of iodotetrazolium on tumor growth was also determined on male mice of the C3H strain bearing a transplantable mammary adenocarcinoma. Four days following subcutaneous transplantation of the tumor, the animals were injected intraperitoneally with 0.5 mg. of nonradioactive iodotetrazolium in 0.1 ml. of water. Injections were con-

tinued five times per week for a total dose of 5 to 15 mg. of iodotetrazolium salt. There was no difference in the external measurements of the tumors in the treated and untreated mice. Ten treated mice died within 15 to 49 days following transplantation, for an average survival of 28 days. The 10 untreated mice died within 16 to 48 days, for an average survival of 29 days.

SUMMARY

Radioiodotetrazolium salt in 0.1-mg. dose, was injected intravenously into normal and tumor-bearing mice. Radioactivity disappeared rapidly from the circulating blood, and was rapidly excreted by the kidney and liver. Transplantable mammary carcinoma and lymphoma contained less radioactivity than most normal tissues.

ACKNOWLEDGMENTS

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COMPLEMENT FIXATION IN ANIMAL NEOPLASIA. I. A STUDY OF TECHNIQUES FOR MEASUREMENT OF THE REACTION IN RABBIT SERUM WITH SPECIAL REFERENCE TO THE TEMPERATURE OF INACTIVATION.[1 2 3]

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In the past, numerous attempts have been made to detect malignant disease by means of complement fixation tests (*1*, *2*, *3*) but unfortunately, although some of these tests appeared to be promising, they were neither very sensitive nor specific. Recent developments in the technique of complement fixation tests themselves and in the methods of fractionation of body tissues and fluids made it seem worth while to reinvestigate the possibility of using this type of test for the diagnosis of human neoplasia.

A study of the reaction in animals bearing a neoplasm of reproducible cell type appeared to offer a logical approach. Since Kidd (*4*, *5*) had obtained positive complement fixation tests with the sera of certain rabbits bearing the transplantable Brown-Pearce carcinoma, this neoplasm was chosen for the first study. Although Kidd had obtained many positive reactions, Cheever (*6*) and Jacobs and Houghton (*7*) observed very few positive tests when the sera of market-breed animals were examined by his technique. Later work of Friedewald and Kidd (*8*) and MacKenzie and Kidd (*9*) indicated that the serum of market-breed rabbits bearing the neoplasm did not contain as much specific complement-fixing antibody as did that of rabbits of the blue-cross variety used in much of their early work.

Since the blue-cross variety of rabbits is now unobtainable, our study was begun with the use of market-breed New Zealand White rabbits. Results of the early tests in which the technique of Kidd [4] was followed as closely as possible confirmed the findings of Cheever and of Jacobs and Houghton. Only very few weakly positive reactions were observed, even

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[2] This work was supported in part by grants from the American Cancer Society and the Damon Runyon Fund.

[3] Presented in part at the 40th annual meeting of the American Association for Cancer Research, Detroit, April 16 and 17, 1949.

[4] To saline dilutions of serum were added two units of complement and an antigen consisting of a partially clarified saline extract of the neoplasm. After incubation for 2 hours at room temperature, remaining complement was roughly measured by the degree of hemolysis resulting from the use of the hemolytic indicator—5 percent sheep cells sensitized with 4 units of hemolysin and incubation for 30 minutes at 37° C. Final readings were made after the tubes had been stored in the ice box overnight.

with specimens obtained four or more weeks after inoculation with the neoplasm—the time at which Kidd had obtained the most positive tests. Although many negative reactions were obtained with 1:10 dilutions of sera, it was noted that in several of the tests final hemolysis occurred more slowly than in tests of normal serum and in the serum and antigen controls. This suggested the possibility that less complement remained in these tests because small amounts had been "fixed."

In early studies of various complement fixation tests (*10*) stronger reactions had been found to occur when fixation was allowed to proceed for a longer time at lower temperatures. Consequently the fixation period and temperature outlined in Kolmer's [5] technique for the Wassermann test were compared with the conditions used by Kidd. Many tests that had been negative by Kidd's procedure now either became frankly positive or showed delayed hemolysis, but occasionally the validity of positive tests was questioned because doubtful or even slightly positive reactions were obtained in similar tests of normal serum.

In both the technique used by Kidd and in the Kolmer test, complete lysis of the designated amount of sheep cells is the end point used in determining the quantities of complement and of hemolysin required for the hemolytic system. Then two such 100 percent complement units are added to the tests and to the controls. A test is considered to be positive only when the amount of complement activity lost during the fixation period (by simple deterioration, because of anticomplementary properties of serum or antigen and/or because of actual complement fixation) exceeds one unit, so that less than one unit remains and, consequently, complete hemolysis is no longer observed. The slower clearing of the 2-hour tests and the positive reactions in the 18-hour tests indicated that small amounts of complement were being "fixed" and that weak reactions were actually occurring. The extent of the reactions could not be easily measured, however, simply by detecting the decreased rate of hemolysis, nor was it feasible to disregard all tests which were accompanied by doubtful or slightly positive controls.

It seemed likely that the reactions could be measured if the actual amounts of complement required for hemolysis in the tests and in the controls could be compared by setting up the tests in the presence of several different amounts of complement. In addition, the use of the end point of 50 percent hemolysis as a measure of complement activity instead of the less accurate 100 percent end point appeared to offer a likely method for the more precise measurement of these rather weak reactions (*12–15*). The present report summarizes our study of this technique and its application to the measurement of these complement fixation reactions. The effects of the temperature at which the sera were inactivated are discussed in some detail.

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[5] Incubation of serum, antigen and two units of complement for 18 hours at 6°–8° C. followed by the addition of 2 percent cells sensitized with two units of hemolysin. The mixture is now incubated at 37° C. and the tests are read 10 minutes after the controls show complete hemolysis (*11*).

MATERIALS AND METHODS

Heavy cell suspensions of the neoplasm carried in New Zealand White rabbits were prepared aseptically by mincing the tissue, forcing it through a tumor press and suspending it in 0.85 percent sodium chloride solution. Adult male animals were inoculated in multiple sites with 0.5-ml. portions of this suspension (usually intramuscularly, intratesticularly, and intraperitoneally). Multiple growths were observed in nearly all of the animals in 1 to 2 weeks, and most of these animals died 3 to 4 weeks after inoculation. However, some of the inoculated animals lived for 3 to 4 months, at which time they were sacrificed.

Reagents

Since the amounts of complement activity remaining, and hence the amounts fixed, were to be measured by means of a hemolytic reaction, an attempt was made to choose reagents which would be most favorable to this hemolytic indicator reaction.

Diluent: All reagents were diluted with 0.85 percent sodium chloride solution containing enough magnesium sulfate to give a final magnesium concentration of 0.01 percent, a concentration said to be approximately the optimum for immune hemolysis (*11*). This magnesium-saline diluent will be termed "saline" in this report. Parallel tests using this diluent and the magnesium and calcium-containing veronal buffer employed by Mayer *et al.* (*16, 17*) showed that, although the final titers obtained were nearly identical, the use of the unbuffered diluent apparently made the conditions more favorable for the hemolytic reaction, since usually slightly less complement was required for hemolysis in both the tests and the controls.

Serum: Rabbits were bled from the heart before inoculation of the neoplasm and at weekly intervals thereafter to provide normal (pre-inoculation) and post-inoculation (tumor) sera. Most of these sera were stored at −20° C. until they were tested. Portions of frozen pools of both post-inoculation and of normal serum were tested daily in order to compare the sensitivity of the test from day to day. The normal rabbit serum pools were composed of the sera of 100 normal market-breed rabbits.

Complement: Commercial lyophilized complement [6] was prepared for use each day by reconstituting with the acetate-boric acid buffer supplied and then diluting 1:10 with ice-cold saline; further serial dilutions, usually 1:20 and 1:100, were prepared from this stock solution.

Antigen: The antigens used were essentially saline extracts of the Brown-Pearce carcinoma, although they may not have corresponded exactly to the preparations used by Kidd. Neoplastic tissue which was not grossly necrotic was saved at autopsy and stored at −20° C. until used. The thawed cold tissue was forced through a monel metal sieve to remove connective tissue, and the remaining material was either ground with sand or treated for 1 minute in a Waring Blendor. The tissue pulp thus obtained was suspended in 4 times its weight of 0.85 percent sodium

[6] Mayer & Myles, Philadelphia, Pa.

chloride and was allowed to stand overnight at 5° C. The 1:5 suspension was centrifuged for 30–60 minutes at 2870 x g and the turbid supernatant fluid was used as the stock antigen. On some occasions this supernate was further clarified by centrifugation for 1 hour in the batch bowl of a Sharples Supercentrifuge with a centrifugal force of about 60,000 x g at the periphery. These antigens were stored at −20° C. and thawed just before use, at which time they were diluted with saline, usually to a final dilution of 1:20 or 1:40. At first antigens were prepared from the neoplastic tissue of individual rabbits, but later neoplasms from as many as 24 animals were pooled and extracted in order to have antigen batches of sufficient amount for comparative tests.

Hemolytic system: Three hundred milliliter portions of sheep blood were collected aseptically in equal volumes of modified Alsever's solution (*18*). Ten or twenty milliliter amounts of the diluted blood were dispensed into sterile rubber-capped serum bottles and stored at 5° C. Cells were not used until they had been stored for at least 4 days, and they were discarded after 6 weeks. The red cells were prepared for use daily by washing six times with saline and diluting to a concentration of 2 percent as measured at a wave length of 545 mμ in a Coleman Jr. Spectrophotometer. When 1 ml. of the 2 percent cell suspension was lysed by 19 ml. of distilled water and 2 drops of dilute ammonium hydroxide, the apparent optical density in a 19-mm. cuvette was 0.250 plus or minus 0.005. Such a suspension contained about 500,000 red cells per cubic millimeter.

A commercial hemolysin found to be rather uniform with respect to its high potency and low agglutinating property was used throughout.[7] Instead of adding a more or less arbitrary number of units of hemolysin, an attempt was made to use the optimum concentration. Kent (*19*) determined the optimum by performing "immediate" (no preliminary incubation) complement titrations by the use of varying hemolysin concentrations. He defined the optimum concentration of hemolysin to be that above which there was no appreciable diminution of the amount of complement required for 50 percent hemolysis. When the optimum was determined under the conditions of our test, however, (in the presence of normal rabbit serum and antigen and using the 18-hour incubation period) usually greater amounts of hemolysin were required. This more nearly optimum concentration was usually a 1:250 dilution of the 50 percent solution of hemolysin in glycerin or a hemolysin dilution of 1:500.

The red cell suspension was mixed with an equal volume of this optimum dilution of hemolysin and the mixture was incubated for 15 minutes at 37° C. to sensitize the red cells.

Technique of the Test

The test used is essentially a complement titration in the presence of serum and antigen. Usually portions of the undiluted sera were inactivated for 30 minutes and then diluted 1:10 or 1:20 with saline. Graduated amounts of complement diluted to 0.40 ml. were added to a series of

[7] Mayer & Myles, Philadelphia, Pa.

tubes containing 0.40-ml. portions of the diluted serum; 0.40-ml. volumes of antigen were then added and the mixtures were incubated at 5° C. for 18 hours. Following this fixation period sensitized cells were added and the tubes were incubated for 30 minutes at 37° C. in order to allow hemolysis to occur. The tubes were then chilled immediately in order to delay any possible continued hemolysis, the unhemolyzed cells were quickly removed by centrifugation and the supernatant fluids were poured into standardized 10 mm. cuvettes. The degrees of hemolysis were estimated from the spectrophotometer readings. Table 1 outlines the technique and shows the usual amounts of complement added in a test. The amount of complement that would have given exactly 50 percent hemolysis in each

TABLE 1.—*Sample protocol of complement fixation test used in this study*

Tube Number	1	2	3	4	5	6	7	8
Diluted test serum--ml--	0. 400	→						→
Complement:								
Dilutions used	1:100	1:100	1:100	1:100	1:20	1:20	1:20	}
Ml. added	0. 100	0. 200	0. 300	0. 400	0. 120	0. 160	0. 200	} 0. 000
Amount as 1:100 dil---	(. 100)	(. 200)	(. 300)	(. 400)	(. 600)	(. 800)	(1. 000)	}
Saline----------ml--	. 300	. 200	. 100	. 000	. 280	. 240	. 200	. 400
Diluted antigen-----ml--	. 400	→						→
	18-hour incubation at 5° C. (fixation period)							
Sensitized sheep red cells----------ml--	0. 800	→						→
	30-minute incubation at 37° C. (hemolysis period)							
	Determine percentages of hemolysis and calculate amount of complement giving 50 percent hemolysis							

titration was read from the typical sigmoid hemolysis curve obtained by plotting the amounts of complement used against the resulting degrees of hemolysis. These amounts were expressed as ml. of a 1 : 100 dilution of complement. In addition to tests of pooled positive and pooled normal sera the following controls were set up daily:

(*a*) An immediate complement titration to measure the activity of the complement actually used.

(*b*) 18-hour complement titrations in the presence of each serum without antigen.

(*c*) An 18-hour titration in the presence of antigen alone.

CALCULATION OF TITERS

To measure the amount of complement fixed, the amount required for 50 percent hemolysis in the test was corrected for:

(1) The amount required in the presence of the test serum alone.

(2) The anticomplementary activity of the antigen in the presence of normal serum.

This latter correction was taken to be the increased complement requirement due to the addition of antigen to the pooled normal serum. The amount of complement fixed in the test was expressed in terms of the 50 percent unit of complement determined in the immediate titration. The number of such 50 percent units fixed was expressed per ml. of undiluted serum.

The following formula expresses the method used for calculating the titer:

$$\frac{TS+A-[TS+(NRS+A-NRS)]}{IC}$$

The symbols in the formula represent the ml. of 1:100 complement required in the following titrations:

TS+A =test serum plus antigen
TS =test serum alone
NRS+A=pooled normal rabbit serum plus antigen
NRS =pooled normal rabbit serum alone
IC =the immediate complement titration required to determine the size of the unit.

It is recognized that the titer expressed in terms of the 50 percent unit determined by an immediate complement titration is a more or less arbitrary value which may not be strictly applicable to the 18-hour test results, but the use of the much greater 18-hour complement unit is probably unjustified because of the rather rapid deterioration of complement which occurs during the 18-hour incubation period in the absence of serum. The experiments to be discussed have shown that not only does the presence of serum influence the amount of complement required in 18-hour tests and controls, but also that the temperature at which the serum is inactivated is of some importance.

The 18-hour complement titrations in the presence of 1:10 dilutions of normal sera inactivated at the customary temperature of 56° C. usually showed a requirement for complement of greater than one immediate unit and sometimes even greater than one 18-hour unit (anticomplementary effect of serum). Sera inactivated at higher temperatures were less anticomplementary, and when the inactivation temperature was raised to 70° C. the requirement was approximately one immediate unit (protective or activating effect of serum on complement?).

Wadsworth (*14*) and Rice (*15*) have made use of the formula $\frac{TS+A}{TS}$ in calculating titers. We have also calculated titers in a similar manner, after correcting for the anticomplementary properties of the antigen and test serum by using the following formula:

$$\frac{TS+A-[TS+(NRS+A-NRS)]}{TS}$$

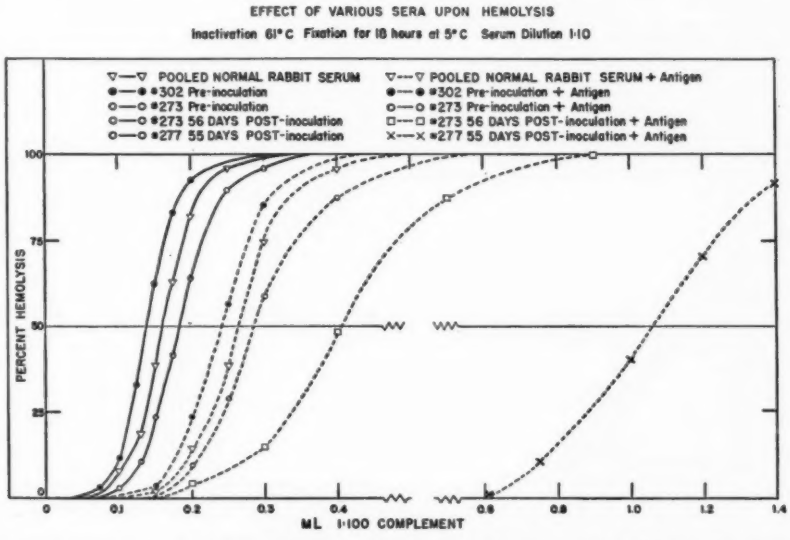
Because of the effect of the inactivation temperature upon the value of TS itself, and the additional differences between individual sera, it seemed

more logical to express the extent of the reaction in terms of the fixed immediate complement unit, IC.

THE EFFECT OF THE TEMPERATURE OF INACTIVATION

Text-figure 1 shows the complement-hemolysis curves obtained in tests of three normal sera and two post-inoculation sera. The first three curves show that the sera in the absence of antigen had varying effects upon the amounts of complement required for 50 percent hemolysis (0.139 ml. for #302 PI to 0.185 ml. for #273 PI and tumor serum #s 273 and 277). Hereafter the term tumor serum (TS) will be used to denote serum from a tumor-bearing rabbit, and PI will denote pre-inoculation serum. The next three curves illustrate the increased requirements for complement upon the addition of the antigen to NRS and to individual pre-inoculation sera. The increased requirements due to the presence of the antigen were nearly identical with the three normal sera. The last two curves show the still greater increases in the amounts of complement required by the tumor sera when mixed with antigen. These extra requirements are taken to be the amounts of complement fixed. Using the formula given previously, the titer of tumor serum #273 was 23, while that of TS #277 was 161.

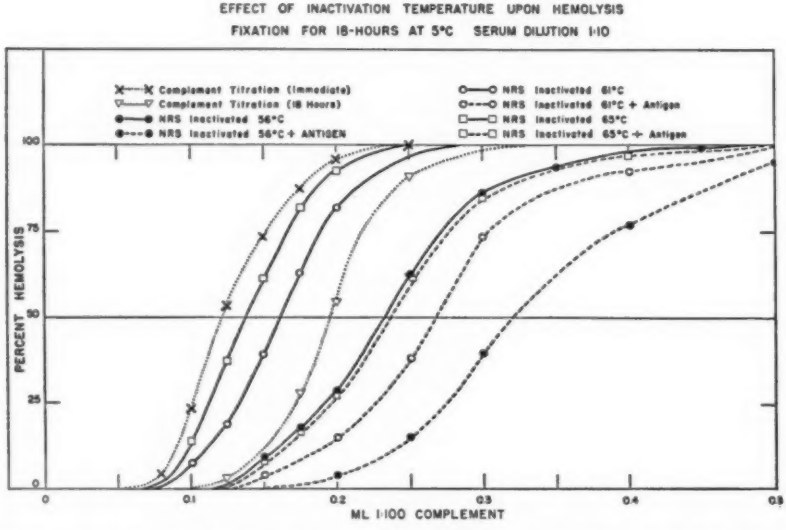
Kidd and Friedewald (*20*) reported the presence of a natural antibody in the sera of nearly all normal adult rabbits. It reacted with saline extracts of Brown-Pearce tumors as well as with similar extracts of normal rabbit tissues. Its effect was no longer noted when the sera were inactivated at



TEXT-FIGURE 1.—Complement-hemolysis curves obtained in tests of 1 : 10 dilutions of three normal sera and two post-inoculation sera. Inactivation for 30 minutes at 61° C.; incubation for 18 hours at 5° C.; antigen Pool II, 1 : 20 dilution.

65° and, since the Brown-Pearce antibody was not destroyed at this temperature, their later tests were made on sera inactivated at 65° C. The effect of the inactivation temperature was also studied by Hazen (*21*). She found that the amounts of complement required for hemolysis, after preliminary 4-hour incubation at 3°–6° with human, guinea pig or rabbit serum, decreased rapidly and then more slowly as the inactivation temperatures were increased from about 53° to 70° C. In complement fixation tests of human syphilitic sera the amount of fixation increased as the inactivation temperature was raised from 53° to about 57° C., while at still higher temperatures the reaction diminished. In view of these reports, sera of normal and of tumor-bearing rabbits were inactivated at various temperatures in order to study the effect of inactivation temperature upon the amount of complement required for 50 percent hemolysis in our 18-hour tests.

Effect of the serum: Text-figure 2 shows the results of one such experiment in which samples of a pool of normal rabbit serum were inactivated at three different temperatures before determining the amount of complement required in the presence of the serum, both alone and with antigen. Titration curves for complement alone titrated both immediately and by the use of the fixation period used in the tests are included for reference. The curves show that the amount of complement required varied with the temperature of inactivation of the serum. Serum inactivated at 65° required the smallest, and that inactivated at 56° C. required the greatest amount. This was true both in the presence and absence



TEXT-FIGURE 2.—Complement-hemolysis curves demonstrating the effect of inactivation temperature upon the amount of complement required for 50 percent hemolysis. Pooled normal rabbit serum (NRS), 1 : 10 dilution; antigen Pool II, 1 : 20 dilution; incubation for 18 hours at 5° C.

of antigen. The increased requirements for complement due to the presence of antigen were nearly identical at the three temperatures.

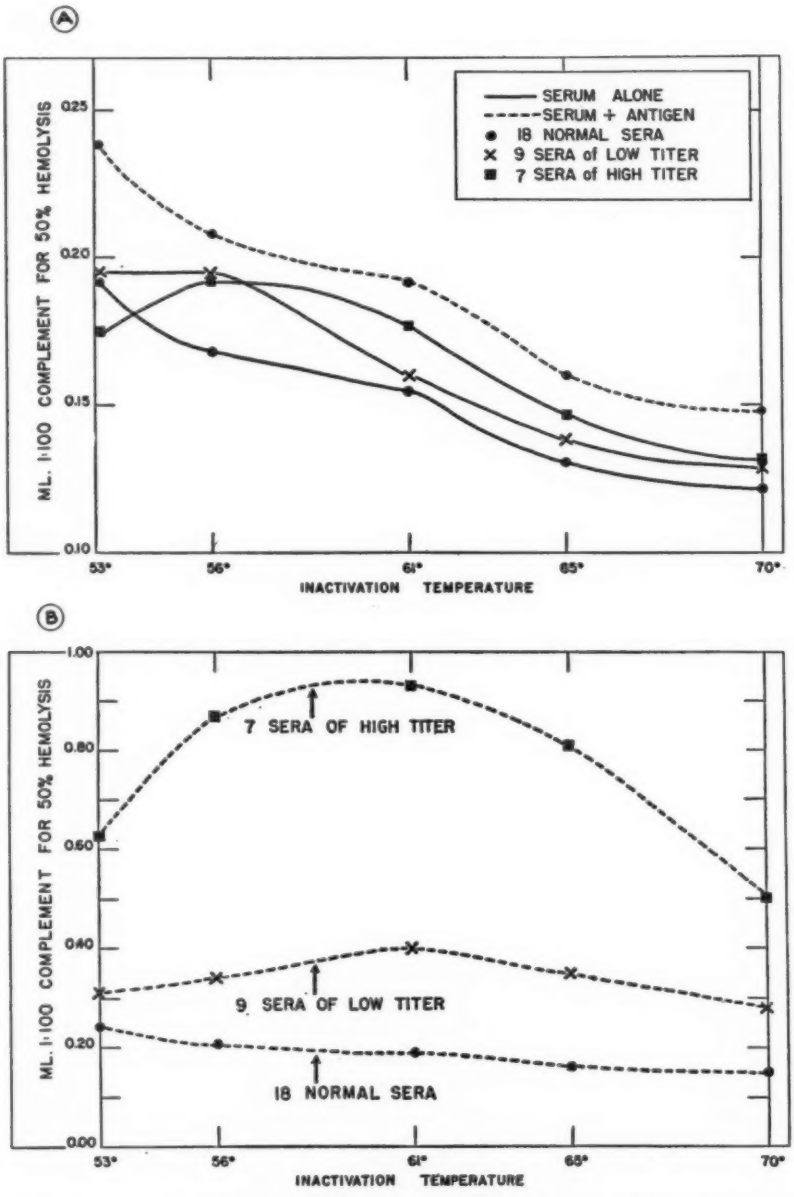
The effects of inactivation temperatures covering a wider range were studied by inactivating both individual and pooled normal and tumor sera at temperatures ranging from 53° to 70° C. Text-figures 3A and B show the average results obtained with 18 normal sera, 9 weakly-reacting tumor sera and 7 more-reactive tumor sera both in the absence and presence of antigen. The average curve obtained with the frozen normal sera alone resembled that described by Hazen, decreasing rather rapidly from the 53° value and gradually leveling off near 70° C. Many of the individual tumor sera gave temperature curves of a different shape, for the values increased from 53° to 56° or 61° C. and then decreased. The peak at 61° C. was usually most noticeable with the more-reactive sera. The different character of these TS curves is reflected in the average curves for the weakly- and strongly-reactive sera shown in text-figure 3A.

Effect of the antigen: The addition of the anticomplementary antigen to the normal serum resulted in a temperature curve which was similar to, but somewhat higher than, that of the normal serum itself (text-fig. 3B). When antigen was added to the tumor sera, however, 12 of the curves showed a marked peak at about 61° while 3 peaks were at 56° and 1 was at 65° C. It can be seen that the average curves of both the weakly- and more strongly-reacting sera showed maxima at approximately 61° C. Inactivation at higher temperatures resulted in a gradual decrease in the requirement for complement.

Since the point of maximum reaction usually was near 61° C., this inactivation temperature was used routinely for detecting reactive sera. The tendency for tumor serum alone to require more complement than normal serum when inactivated at this temperature is one reason for the particular correction used in calculating the titer. The assumption is made that the anticomplementary properties inherent in the tumor serum are not appreciably affected by the presence of an anticomplementary antigen. The correction, then, is taken to be the value TS plus the "anticomplementariness" of the antigen in the presence of normal serum, rather than the comparable value for normal serum, NRS+A.

Effect of serum-antigen ratio: The tests illustrated in text-figure 3 were made with a constant serum-antigen ratio (1:10 serum and 1:40 antigen). The temperature effect was further studied by varying the dilutions of serum and antigen. Several individual and pooled positive sera of varying reactivity were inactivated at temperatures ranging from 53° to 70° C., and a number of dilutions were tested with a constant amount of antigen. In similar experiments a constant serum dilution was tested with a series of antigen dilutions. In these tests with varying serum-antigen ratios the peak reaction again was obtained with serum inactivated at 61° C.

Effect of dilution before and after inactivation: It was noted that all sera became more turbid when heated to 65°–70° and that certain tumor sera coagulated at 70° C. This coagulation did not occur when the sera were



TEXT-FIGURE 3.—Average inactivation temperature effects of 1 : 10 normal and positive sera. Incubation for 18 hours at 5° C.

A.—Effects of serum alone.

B.—Effects of serum plus antigen (1 : 20 or 1 : 40).

diluted before inactivation. The effect of this prior dilution upon the amounts of complement required at different inactivation temperatures was studied by testing two portions of each of several sera, one being inactivated before, and the other after dilution. The titers obtained with 1:10 dilutions of TS #255 when inactivated before and after dilution were as follows: at 56°, 60 and 63; at 61°, 89 and 93; at 65°, 90 and 95; at 70° C., 60 and 74. The average titers obtained at 61° and 70° with the three other sera tested in the same way were as follows: at 61°, 92 and 96; at 70°C., 50 and 76. It appears that nearly identical titers are obtained with sera diluted either before or after inactivation at temperatures of from 56° to about 61° C. Undiluted tumor sera inactivated for 30 minutes at 70° C. require definitely less complement and give lower titers than do the same sera diluted 1 : 10 before inactivation.

Effect of storage of sera: In order to study the effect of storage of sera in the frozen state upon their reactivity and upon the temperature effect, parallel tests were performed on certain sera when they were fresh (the day the blood was drawn) and after storage at −20° to −30° C. for at least a week. Text-figure 4 shows the average inactivation temperature effect observed in tests of fresh and frozen sera of 3 animals taken before and 21-32 days after inoculation. The fresh serum-antigen mixtures, both pre- and post-inoculation, required definitely less complement at 53° and 56°, but the fresh and frozen values were nearly identical at 61° and 65° C. Three of the average curves show peak values near 61° C., while the frozen pre-inoculation (normal) curve resembles the decreasing normal serum-antigen curve shown in text-figure 3. Also included in text-figure 4 are the average curve obtained from tests of 11 more-reactive fresh tumor sera and that resulting from tests of 13 frozen tumor sera giving titers of the same order of magnitude. Again it can be seen that tests of fresh sera tend to require relatively less complement at 53° and 56°, that both curves have nearly identical maxima near 61°, and that they are rather similar from 61° to 70° C. Definitely less reaction is obtained with sera inactivated at 70° C.

A total of 48 sera was tested with the same antigen preparation, both fresh and after storage in the frozen state for 1 to 3 weeks. Statistical analysis of the results showed that the 61° C. titers (range 0–151) were not significantly affected by the storage. Similarly, the effect of storage (8–17 weeks) of 19 sera by refrigeration was compared with that of storage for the same periods of time in the frozen state. The titers obtained in tests of refrigerated samples of sera were significantly ($P<0.05$) greater than those obtained in simultaneous tests of the frozen samples. Since the titers obtained with corresponding frozen and fresh sera did not differ, storage at −20° C. was the usual method of preservation.

Effect of a longer inactivation period: Sera were usually inactivated by heating for 30 minutes. The possibility that a longer inactivation period might give different temperature effects was also investigated. Duplicate samples of fresh and frozen normal and tumor sera were inactivated for 30 and 45 minutes at each of the temperatures studied. They were

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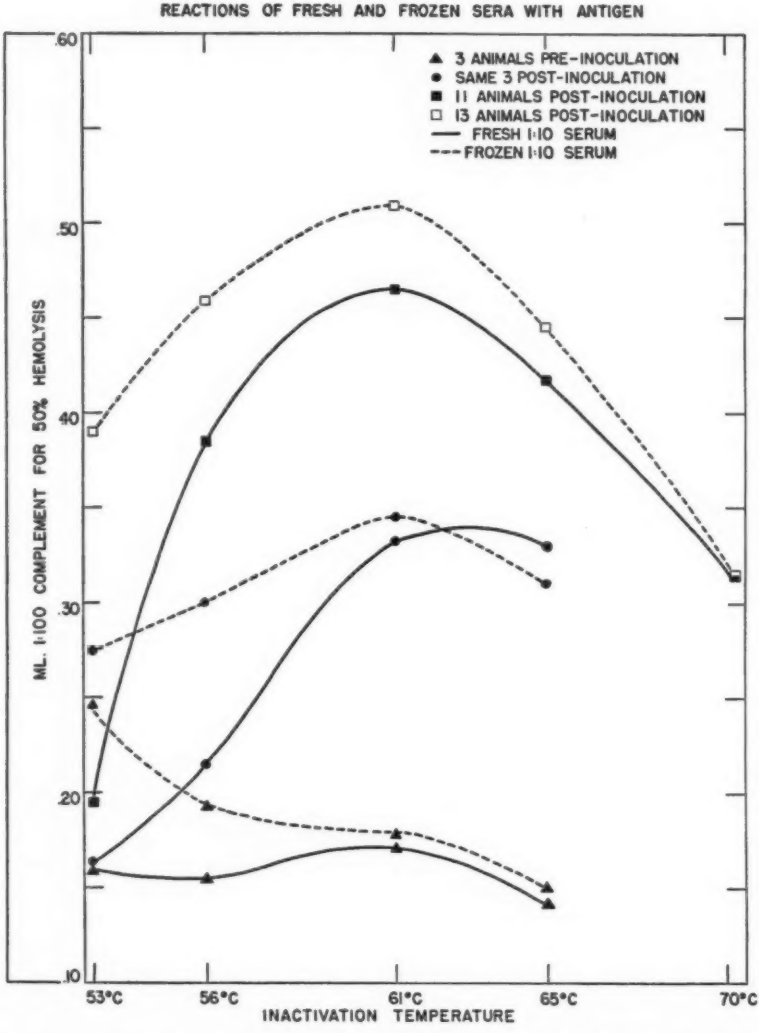
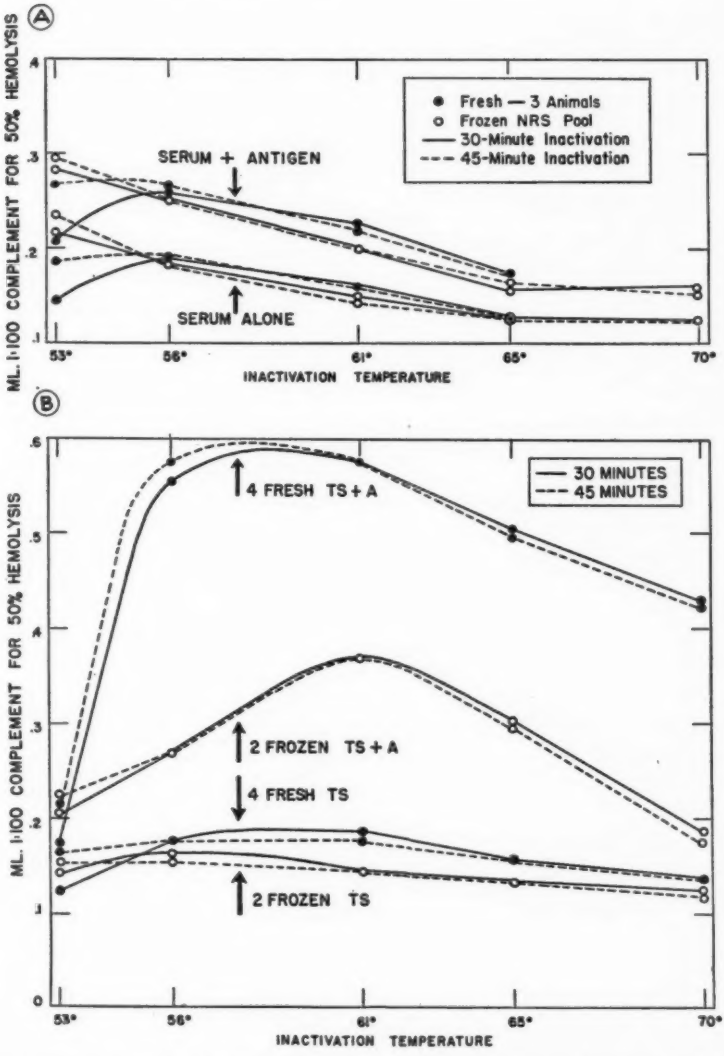


TEXT-FIGURE 4.—Temperature effects of fresh sera plus antigen and of identical or comparable frozen sera plus antigen. Antigen dilution 1 : 40; incubation for 18 hours at 5° C.

diluted 1 : 10 and the 18-hour complement requirements were determined both in the presence and absence of antigen. The results obtained with the sera inactivated at all five temperatures are shown in text-figures 5A and B. The additional time of heating at 53° C. definitely increased the requirement for complement for each serum, both alone and in the presence of antigen, but the greatest increase was found with fresh serum. There was also a tendency for fresh sera to require slightly more complement



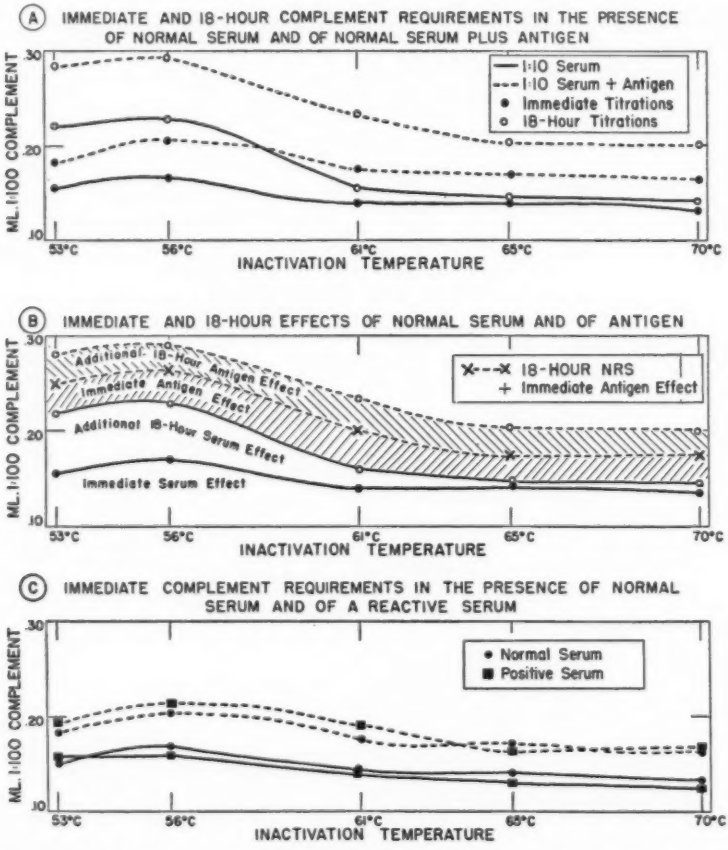
TEXT-FIGURE 5.—Influence of a longer inactivation period upon temperature effects of fresh and frozen 1 : 10 sera. Incubation for 18 hours at 5° C.

A.—Normal sera with and without antigen 1 : 40.
B.—Tumor sera with and without antigen 1 : 40.

when inactivated 45 minutes at 56° C., but the additional time at higher temperatures had a negligible effect upon either fresh or frozen sera, alone or with antigen. Evidently inactivation for 30 minutes at 53° is usually insufficient to destroy all of the hemolytic properties of fresh rabbit serum, and this is sometimes true at 56° C. Apparently storage,

even in the frozen state, results in the loss of some hemolytic activity, since under these conditions less heating at 53° C. is required to destroy it. No appreciable difference was noted between the effects of the two inactivation times at temperatures of over 56° C. For this reason inactivation for 30 minutes was used as a standard procedure.

Immediate versus 18-hour temperature effect: An attempt was made to determine whether the inactivation temperature effect was an immediate action upon the hemolytic system or whether it developed during the 18-hour incubation period. Samples of a frozen NRS pool and a frozen reactive serum #265 were inactivated at temperatures of 53°–70° C. The complement requirements in the presence of serum alone and in mix-



TEXT-FIGURE 6.—Immediate serum and antigen effects and their increase during the 18-hour incubation at 5° C. Serum 1: 10; antigen Pool II 1: 40.

A.—Immediate and 18-hour complement requirements with NRS alone and in the presence of antigen.

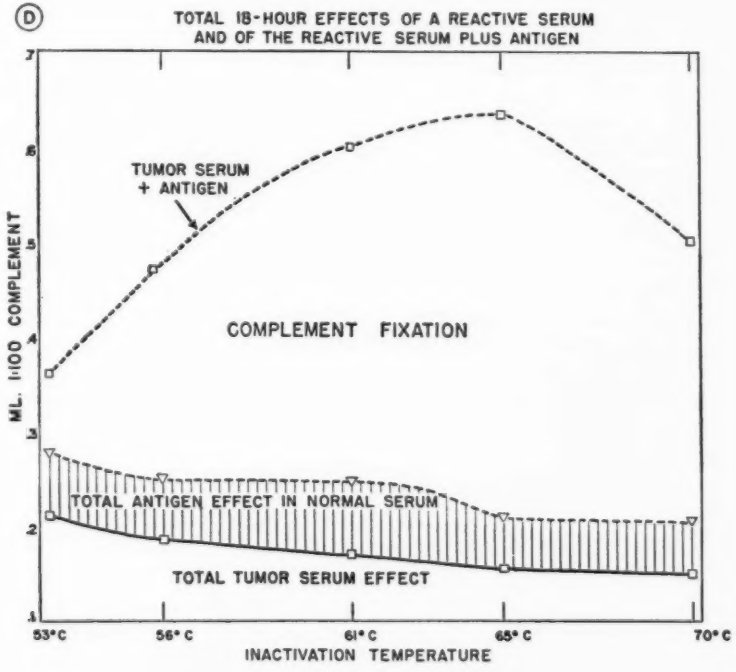
B.—Immediate and 18-hour effects of NRS and of antigen.

C.—Immediate effects of NRS and of TS #265 with and without antigen.

tures with antigen were determined both at once and by the use of the usual 18-hour fixation period.

The temperature curves shown in text-figure 6A indicate that the 1:10 dilutions of the normal serum showed some temperature effect when the hemolytic system was added immediately: slightly more complement was required with serum heated at 53° or 56° C. than with that heated at higher temperatures. The presence of the slightly anticomplementary antigen resulted in a similar, but slightly higher, temperature curve. At 18 hours the complement requirement of normal serum was definitely increased (anticomplementary) at 53° and 56°, while at 65° and 70° C. the immediate and 18-hour values were nearly identical. The presence of the antigen during this 18-hour period also resulted in an additional requirement for complement (more anticomplementary) at each temperature. Consequently, the 18-hour serum plus antigen curve was rather similar to, but definitely higher than, that of the serum alone.

In text-figure 6B these values have been rearranged in order to demonstrate both the immediate and the 18-hour effects of the serum and of the antigen in the presence of normal serum. The two lower temperature curves are those of NRS alone at the two times. The additional complement requirements due to the immediate effect of the antigen (immediate



TEXT-FIGURE 6.—Continued.

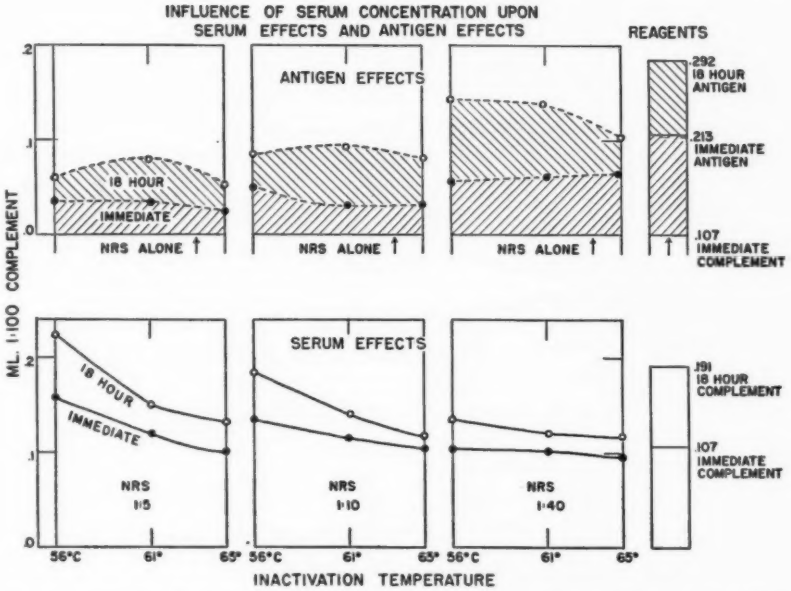
D.—Total 18-hour effects of TS #265 with and without antigen.

NRS+A−NRS) have been added to the total 18-hour serum requirements to outline the total serum effect plus the immediate antigen effect. The remaining effect (difference from 18-hour NRS + A) then should represent that due to the presence of the antigen during the 18-hour incubation period. It is apparent that both serum and antigen have immediate anticomplementary effects and that these increase upon continued contact with complement. This immediate serum effect appears to be truly a property of the inactivated serum, and not one which develops during the 30-minute period of incubation with complement and sensitized cells. In another experiment, samples of a normal serum pool were inactivated at three different temperatures and the immediate complement requirements were determined in duplicate. Duplicate portions of the same inactivated samples were incubated for 30 minutes at 37° C., then sensitized cells were added and the mixtures were again incubated in the usual manner. This preliminary incubation at 37° C. did not increase the requirements for complement.

The immediate temperature curve for the 1: 10 dilution of the particular tumor serum tested happened to resemble that of the normal serum (text-fig. 6C). In addition, the presence of the antigen had nearly identical immediate effects upon the two sera. Text-figure 6D shows that 18-hour incubation of this particular tumor serum also produced a slightly greater complement requirement at all inactivation temperatures, and that the 18-hour TS curve differed slightly in shape from that of the NRS curve. In the presence of antigen, however, there was a very much greater requirement for complement at each temperature tested, the greatest being at 65° C. for this particular tumor serum.

This text-figure also demonstrates the values used in calculating the titer. Since sera differ in their anticomplementary properties, a correction is made for the complement requirement in the presence of the particular serum tested. The immediate effects of the antigen upon the hemolytic system have been shown to be nearly identical in the presence of similar concentrations of normal serum and tumor serum (text-fig. 6C). Consequently, the total 18-hour nonspecific effect of antigen in the presence of normal serum (NRS+A − NRS) is considered to be a close approximation of its nonspecific effect in the presence of tumor serum. The total complement requirement in the presence of tumor serum and antigen minus the sum of TS and NRS+A − NRS is considered to be the amount of complement fixed.

Effect of serum concentration: The anticomplementary effects of serum and of antigen were further studied by determining the complement requirements of several dilutions of NRS inactivated at 56°, 61°, and 65° C., both alone and with antigen. The results shown in text-figure 7 demonstrate that, again, inactivation of normal serum at higher temperatures decreased its anticomplementary property, and that this property also was decreased by dilution of the serum. In addition, the serum tended to prevent the deterioration of complement during incubation, for the 18-hour complement requirements, even at a dilution of 1:40, were



TEXT-FIGURE 7.—Influence of serum concentration upon serum effects and antigen effects. Antigen Pool VIIa 1:40.

definitely less than the value of the 18-hour complement titration in the absence of serum. Tumor serum Pool VIII tested in the same manner had a similar, but definitely greater, anticomplementary effect which also was decreased by dilution. This tumor serum also had a definite protective effect upon complement.

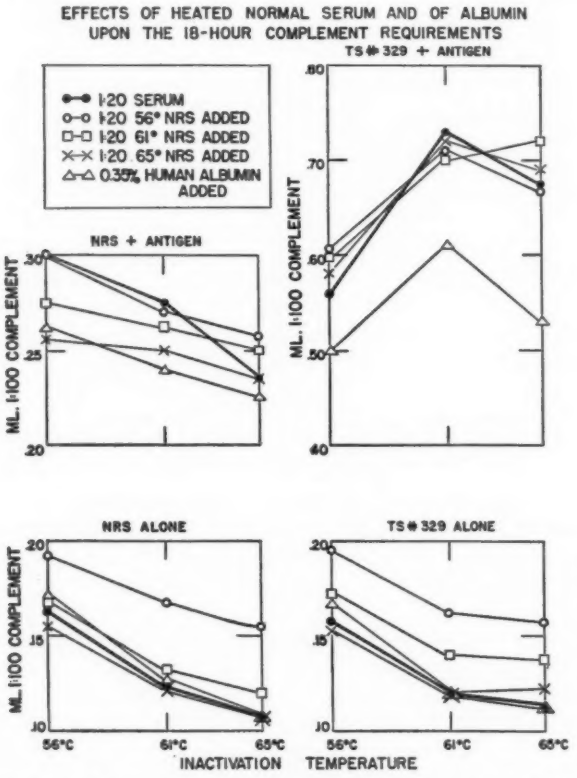
The immediate effect of the rather anticomplementary antigen preparation varied but little with the inactivation temperature of the NRS, but it tended to increase as the serum concentration decreased. Upon further dilution of the serum its effect would approach that of antigen in the absence of serum. The 18-hour antigen effect appeared to vary slightly with the temperature used, and it also increased upon dilution of the serum.

It is obvious, then, that the effect of the particular dilution of the particular inactivated serum being tested cannot be ignored. In addition, the nonspecific effect of the antigen in the presence of the same dilution of inactivated normal serum must be considered. This latter effect appears to be one upon some component or components of the hemolytic system, rather than a true complement fixation, for it is diminished in the presence of increased amounts of serum.

Possible inhibition of fixation by NRS inactivated at 56° C.: The fact that usually less complement is fixed in tests of TS inactivated at 56° than in similar tests of the serum inactivated at 61° C. might be explained by the presence of a thermolabile inhibitor in the tumor serum. If such an inhibitor were present in 56° C. NRS, the presence of this serum in the

test during the 18-hour incubation period should decrease the amount of complement fixation. This possibility was investigated by setting up tests and controls of 1:20 dilutions of TS #329 inactivated at 56°, 61°, and 65° C. To similar tests and controls were added equivalent amounts of NRS inactivated at 56° C. For comparative purposes the effect of adding NRS inactivated at 61° or at 65° C. and the effect of 0.1 ml. of 1.4 percent human albumin [8] were also studied.

The results illustrated in text-figure 8 show that upon the addition of the anticomplementary 56° C. NRS definitely more complement was required in titrations of both 1:20 NRS and 1:20 TS alone. The addition of 61° NRS resulted in temperature curves which were also similar to, but only slightly higher than, the curves without added NRS, while 65° C. NRS and human albumin had little if any effect. The total effect of adding NRS to the 1:20 NRS+A control titrations is the result of two effects of heated NRS: (1) its anticomplementary properties and, (2) its ability



TEXT-FIGURE 8.—Eighteen-hour incubation of NRS, NRS+A, TS and TS+A with and without added albumin or heated NRS. Antigen Pool VIIa 1:20.

[8] Prepared by dilution of human albumin, 25 percent, E. R. Squibb and Sons, New York.

to reduce the "anticomplementariness" of the antigen (text-figure 7). Thus the presence of 56° C. NRS resulted in complement requirements but little greater than the original 1:20 NRS+A requirements. NRS heated to higher temperatures and human albumin actually tended to lower the amount of complement required for hemolysis.

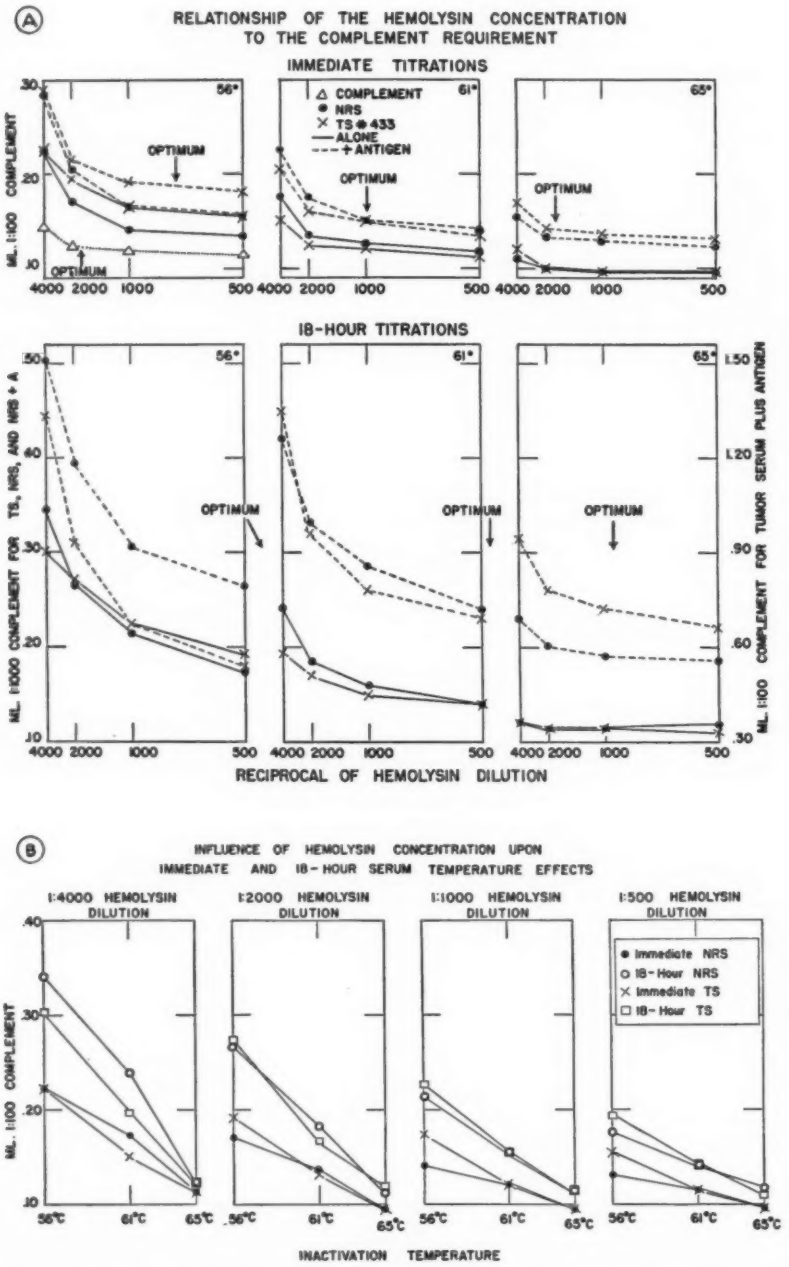
In the complement fixation tests themselves (TS+A), the addition of NRS inactivated at any of the three temperatures had no appreciable effect. Added 56° C. NRS did not lower the complement requirements as might have been expected had it contained an inhibitor. There is still the possibility that 56° C. tumor serum itself contains a substance which inhibits the complement fixation reaction. The addition of human albumin did lower the complement requirements, giving a lower temperature curve which showed maximum reaction at 61° C. The albumin may have inhibited the complement fixation itself, altered the antigen, or supplied some limiting component of complement or hemolysin, with the result that less complement was required to produce 50 percent hemolysis in the tests.

Effect of hemolysin concentration: We have considered the optimum concentration of hemolysin to be that above which there is no appreciable diminution of the amount of complement required for 50 percent hemolysis under the conditions of the test (18-hour NRS+A, page 99). This optimum concentration may be influenced by the individual serum, by the temperature at which it is inactivated, by the 18-hour incubation period and by the particular antigen being used.

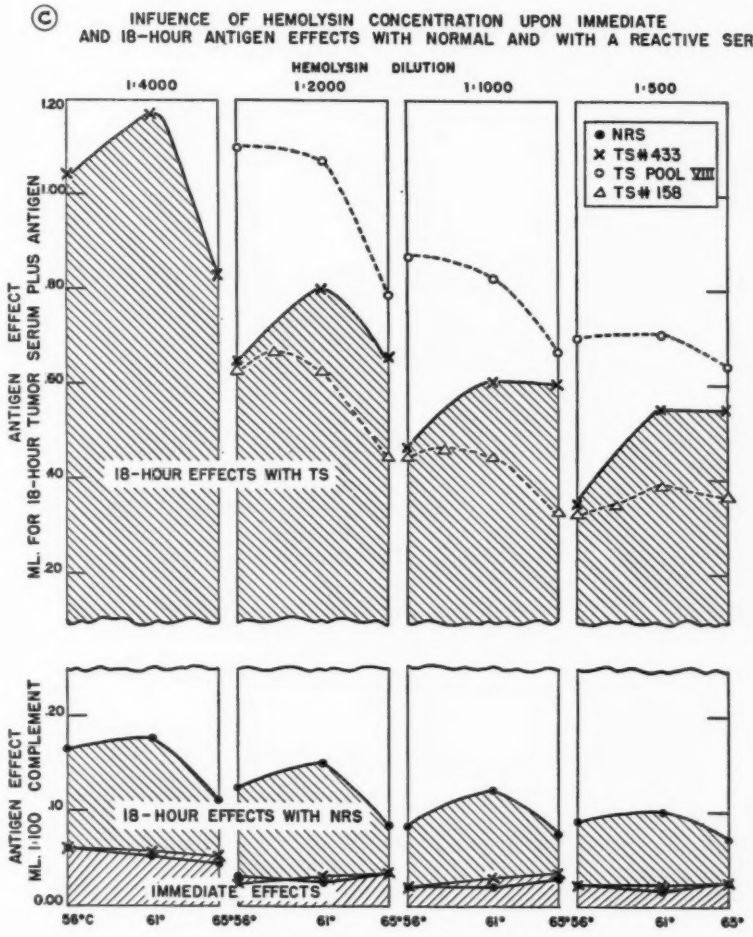
Text-figure 9A illustrates the results obtained when controls and tests of 1:10 dilutions of TS #433 were titrated by the use of cells sensitized with hemolysin at dilutions of 1:500 to 1:4,000. The optimum hemolysin concentration as determined by the use of immediate complement titrations (*19*) was approximately a 1:2,000 dilution. Immediate titrations in the presence of 65° NRS and TS both with and without antigen showed that the optimum dilution was still 1:2,000; with 61° serum it was near 1:1,000; and with 56° C. serum it was near 1:750. In the 18-hour serum titrations the hemolysin requirements were increased, and there was a tendency for still greater requirements in the presence of antigen. The optimum dilutions for 18-hour NRS+A and TS+A were approximately as follows: 1:1,000 for 65° serum, 1:500 for 61° serum, and somewhat less than 1:500 for 56° C. serum.

In text-figure 9B and C these data have been rearranged in order to demonstrate the effect of suboptimum amounts of hemolysin upon the serum temperature curves and upon the immediate and 18-hour antigen effects. As the hemolysin concentration was increased, the complement requirements of the serum without antigen tended to decrease, especially when the lower inactivation temperatures were used (text-fig. 9B). The immediate antigen temperature effects with NRS and TS and the 18-hour effects with NRS did not change in character, although they tended to decrease as the hemolysin concentration increased (text-fig. 9C).

The curve showing the effect of antigen on TS #433 with 1:4,000 hemoly-



TEXT-FIGURE 9.—Relationship of the hemolysin concentration to the complement requirement. Serum 1 : 10; antigen Pool VIIa 1 : 40.



TEXT-FIGURE 9.—Continued.

A.—Change in optimum hemolysin concentration with serum inactivation temperature and upon incubation for 18 hours at 5° C.

B.—Immediate and 18-hour NRS and TS inactivation temperature effects at different hemolysin concentrations.

C.—Immediate and 18-hour antigen effects with NRS and TS inactivatetd at several temperatures. TS #158 with antigen #112 1:40.

sin shows the usual peak value at 61° C. Doubling the amount of hemolysin resulted in a definitely lower curve which still showed maximum reaction at 61° C. Further increases in the hemolysin concentration again resulted in relatively greater decreases in the complement requirements when the serum was inactivated at the lower temperatures, so that the final temperature curve obtained with 1:500 hemolysin showed a peak complement requirement at about 63° C. for this TS. Similar curves obtained on other occasions with 1:10 dilutions of TS Pool VIII and TS #158 are also shown for comparison. Again with these two sera, as the hemolysin concentration was increased the complement requirements, due to the presence of antigen, decreased more rapidly with the lower inactivation temperatures. With the optimum hemolysin dilution of 1:500 these sera showed the greatest requirements for complement when inactivated at 61° C.

When less than the optimum amount of hemolysin is used the complement requirements are increased, especially with sera inactivated at the lower temperatures. This tendency is more pronounced with TS+A, so that the apparent reactions (titers) are actually greater under these conditions. In the region of the optimum hemolysin concentration, then, the values of the controls, the tests, and the titers are lower and less dependent upon small variations in the concentration of hemolysin. For this reason the optimum concentration for 61° NRS+A was used for routine tests of individual sera inactivated at 61° C.

DISCUSSION OF THE INACTIVATION TEMPERATURE EFFECT

The observation (text-figs. 2 and 3) that mixtures of antigen and frozen or refrigerated sera from normal rabbits required more complement when inactivated at 56° than at 61° or 65° C. agrees in general with the findings of Kidd and Friedewald (*20*). However, it would seem that their reports of positive reactions with normal serum inactivated at 56° and negative reactions at 65° C. might be due to the gradual decrease in the amount of complement required as the serum was heated to higher temperatures, rather than to a sharply defined destruction of a true "natural antibody." The use of a suboptimum amount of hemolysin would accentuate this temperature effect. As a matter of fact, the change in the complement requirements of mixtures of antigen and previously frozen normal serum with the change of inactivation temperature has been shown to be essentially a property of the serum. The additional effect of the antigen, both immediate and during the 18-hour incubation period, was nearly constant regardless of the inactivation temperature of the normal serum with which it was mixed.

The significance of the different character of the temperature curves obtained upon mixing antigen with normal serum and with reactive serum is not known, but other workers have obtained evidence of certain rather definite changes which appear in serum heated at about 60°–65° C.

Du Noüy (*22*) heated serum at several temperatures and studied the resulting changes in various physical-chemical properties. The viscosity

was found to decrease until the temperature reached 56°–58° C., but heating at still higher temperatures produced irreversible progressive increases in viscosity. Similarly, changes beginning at about 56°–60° C. were noted in such properties as rotatory power, absorption and scattering of light, and stability and sedimentation of the globulins. He concluded that there are profound modifications in the structure of the proteins and of the "lipo-protidic" complex at about 55°–56° C.

Takenomata (*23*) stated that normal rabbit serum fixed complement with certain bacterial cultures and filtrates, peptone, *etc.*, and that this phenomenon was more pronounced when the preliminary incubation was carried out at 37° rather than at 0° C. This complement "fixation" was prevented by heating the sera to 62° C. before testing.

Kleczkowski and Bawden (*24, 25, 26*) and van der Scheer *et al.* (*27*) have shown that new components were formed by heating horse or rabbit serum at temperatures of about 60° C. or higher. These components were produced at the expense of the albumin and the globulins, including the gamma globulins. Certain antibody-protein complexes so formed then had new properties, apparently depending upon their composition. They combined with antigen, but the resulting complexes were now insoluble or they failed to fix complement. Kleczkowski reported that flocculating antibodies of rabbit antisera were less altered by heat when in saline solution than when in serum, presumably because of the decreased concentration of albumin and the consequent decrease of complex formation. Our duplicate tests with serum samples diluted before and after inactivation showed that the same effect may be observed in complement fixation tests. The preservative effect of 1:10 dilution with saline was marked when the serum samples were heated at 70°, and in the one experiment some difference was noted with samples diluted before and after heating at 65° C. Heating at 61° C. for 30 minutes did not alter the reacting serum components enough to cause any measurable difference in samples diluted before and after heating.

Further evidence of protein interaction in rabbit serum heated at 60°–62° C. was offered by the recent observations of McCrea (*28*). He reported that many normal and immune rabbit sera contained a substance inhibitory to the virus agglutination of chicken erythrocytes. The inhibitory titer was decreased by inactivation for 30 minutes at 56°, but it was destroyed completely in 20 minutes at 62° with but little loss of the specific titer; in fact, heating at 62° C. for an additional hour reduced the specific titer by only 5 percent, although higher temperatures were more harmful. Both specific and nonspecific inhibitors were salted out of the serum in the gamma globulin fraction, and the effects of heat upon them were the same as those upon the sera from which they were prepared.

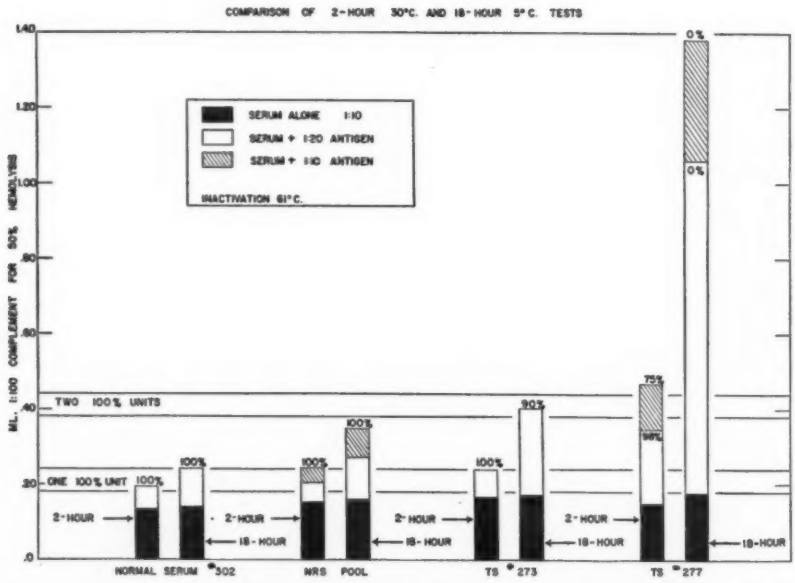
Evidently the heating of rabbit sera for 30 minutes at about 61° C. or higher brings about a number of protein interactions, presumably largely between albumin and gamma globulins. It is known that gamma globulins are anticomplementary (*29*), *i. e.*, they inhibit the hemolytic complement fixation reaction by which complement amounts are measured.

Consequently, the reduction in the complement requirement of serum upon heating at higher temperatures is not remarkable. It is possible that a relatively thermostable component of TS tends to inhibit the complement fixation reaction in serum inactivated at 56° C. and that this inhibitor is destroyed at higher temperatures. Thus larger amounts of complement would be fixed in sera heated at about 61° C. The decreased complement fixation observed upon inactivation at 65° C. or higher is presumably due to the progressive alteration of the reacting substance by heat.

COMPARISON OF 2-HOUR, 30° C. AND 18-HOUR, 5° C. COMPLEMENT FIXATION TESTS

When Kidd's 2-hour test was used in our early work, frequently the only evidence of complement fixation had been a delayed appearance of hemolysis in the tests. When the 18-hour Kolmer-type test was used many tumor sera gave positive tests, but occasionally the controls did not show complete hemolysis. In order to compare the procedure described in this paper with 2-hour and 18-hour 100 percent hemolysis tests, certain sera were examined by Kidd's 2-hour test, by the same technique using 18-hour incubation, and by the described procedure using both fixation periods.

Text-figure 10 shows some of the results obtained with sera inactivated at 61° C. when examined by the four procedures. (The results obtained by use of the described technique are also shown in text-fig. 1.) The



TEXT-FIGURE 10.—Examination of normal and tumor sera by Kidd's 2-hour test, by the same technique using 18-hour incubation, and by the described procedure using both fixation periods. Serum 1 : 10; antigen Pool II.

heights of the vertical bars indicate the amounts of complement required for 50 percent hemolysis both for serum alone and in the presence of one or two amounts of antigen. The percentages recorded at the top of each bar are visual estimates of the degrees of hemolysis observed in the 100 percent tests. Because of the large amounts of complement used in these tests, the readings were made as soon as the controls showed complete hemolysis in order to detect delayed hemolysis in the tests when it occurred. The hemolysis period used was approximately that prescribed by Kolmer and was much shorter than that prescribed by Kidd. Some definitely positive 18-hour tests were obtained, but there were fewer instances of delayed hemolysis in the 2-hour tests. As a matter of fact, further hemolysis did occur in the 2-hour tests and most of these weak reactions finally became definitely negative. The amounts of complement required for 50 percent hemolysis in our procedure have no direct relationship to the size of the 100 percent units used because the two systems differ with respect to the concentrations of cells and hemolysin used, the composition of the diluent, and the methods of reading the results. For these reasons the horizontal bars representing the addition of one and two 100 percent units of complement have been placed in more or less arbitrary positions simply to illustrate the differences between the results obtained under the particular circumstances mentioned. The unknown excess amounts of complement present in these 100 percent hemolysis tests are measured by the distances from the tops of the vertical bars to the horizontal two-unit bar. With the described procedure utilizing the 50 percent hemolysis end point there are no unknown excess amounts of complement, small differences between the amounts of complement required in tests and controls can be measured, and, hence, weak reactions can be detected and measured.

It can be seen that some of the sera were more anticomplementary than others, that the use of more of the anticomplementary antigen required the use of more complement, and that the longer incubation period accentuated this effect even in the presence of normal serum. Most of these effects were not detected by the two-unit methods because of the relatively large excess of complement added. The use of a more anticomplementary serum or antigen, a smaller amount of protective serum, a lower inactivation temperature, or less hemolysin might result in only partial hemolysis because of the greater requirements for complement. Some of these factors probably explain the incomplete hemolysis observed in some of the controls in our earlier experiments with the Kolmer-type test (inactivation at 56° C., suboptimum hemolysin concentration, *etc.*).

It is concluded that increasing the time allowed for complement fixation to occur increases the amount of fixation. The performance of the tests in the presence of different amounts of complement permits calculation of the amounts required for exactly 50 percent hemolysis. The comparison of these amounts with those required in the proper controls permits the detection and measurement of complement fixation.

When this procedure was applied to sera inactivated at the approxi-

mate optimum temperature of 61° C., many positive tests were obtained with New Zealand White rabbits bearing the Brown-Pearce carcinoma.

SUMMARY

Complement titrations have been performed by determining the amounts producing 50 percent hemolysis of the added sensitized sheep cells. These amounts have been expressed as ml. of a 1:100 dilution of complement. Complement fixation occurring during an incubation period of 18 hours has been detected by comparing the amount of complement required in titrations performed in the presence of test serum and antigen with the amounts required in the appropriate controls. Many positive reactions have been obtained by incubating sera of New Zealand White rabbits bearing the Brown-Pearce carcinoma with saline extracts of the neoplasm. Positive tests were obtained with several sera giving negative reactions by Kidd's method.

Both antigen and inactivated frozen serum had immediate effects upon the amounts of complement required for hemolysis; these anticomplementary effects increased during the 18-hour incubation period. The effect of the serum varied with the temperature at which it had been inactivated: at 56° it was considerable while at 65°–70° C. it was negligible. The effect of the antigen was partially inhibited in the presence of normal serum and it varied but little with the serum inactivation temperature. In the presence of reactive serum, however, the effect of antigen was greater and it varied markedly with the serum inactivation temperature; usually maximum complement fixation occurred in tests of serum inactivated at 61° C. These effects were observed at several concentrations of serum and of antigen as well as at several serum-antigen ratios. These properties must be considered in evaluating the reaction.

Titers have been calculated by subtracting from the amount of complement required in the presence of test serum and antigen the sum of the following: (1) the amount required with test serum alone and, (2) the amount required due to the anticomplementary effect of the antigen in the presence of normal serum. Titers have been expressed as units of complement fixed per ml. of test serum.

Some of these temperature effects tended to vary qualitatively and/or quantitatively when suboptimum amounts of hemolysin were used in the final hemolytic reaction. Consequently, the calculated titers also are dependent upon the hemolysin concentration used.

A study was made of the influence of certain technical factors upon both complement fixation and the inactivation temperature effect. These factors were storage of the serum, dilution of the serum prior to inactivation, and inactivation for extended periods of time.

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INFLUENCE OF OVARIAN FUNCTION ON INCIDENCE OF RADIATION-INDUCED OVARIAN TUMORS IN MICE.[1] [2]

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INTRODUCTION

Tumors have long been known to develop in the ovaries of mice exposed to roentgen irradiation (*1–5*). More recently similar neoplasms have been observed in nonirradiated ovaries grafted into the splenic or pancreatic tissue of gonadectomized rats and mice (*6–8*). The histologic appearance and biologic behavior of these neoplasms have been described by Furth and his co-workers (*2, 9–12*) and others (*13*).

The mechanism by which irradiation leads to the development of ovarian tumors has attracted considerable interest. Theoretically, it is possible that such tumors may result from the *systemic* effect of whole-body irradiation, or from *local* action upon the ovary, or from both. The local ovarian response to suitable doses of radiation is a dual one. It involves such *direct* effects as the destruction of ova and follicles, as well as a diminished production or secretion of estrogenic hormone with secondary, *indirect* disturbances in endocrine interrelationships. Since both are sequelae of the same stimulus, experimental attempts to isolate and evaluate each of these effects independently are necessarily attended by serious difficulties.

A number of investigators have presented evidence indicating that the development of tumors in intrasplenic ovarian grafts is at least partially the result of a hormonal imbalance (*6, 14–16*). It has been postulated that estrogens secreted into the portal circulation by the ovarian grafts are inactivated by the liver, with presumptive depletion of systemic circulating estrogens and secondary hypersecretion of pituitary gonadatropic hormone. Prolonged stimulation by this hormone is thought to result in hyperplasia and ultimate neoplasia of the ovarian grafts.

In earlier studies, Li, Gardner, and Kaplan (*17*) failed to obtain definite evidence that a similar mechanism is involved in the induction of such tumors by irradiation. More recently, evidence for such an indirect mechanism was presented by Lick, Kirschbaum, and Mixer (*18*). They found that local irradiation of a single ovary yielded a neoplasm in that ovary only when the opposite ovary was excised or also irradiated. This paper presents the confirmatory results of a quite different experiment designed to study the same problem.

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[2] This work was initiated in the Section of Biophysics, National Cancer Institute, Bethesda, Maryland, and carried to completion in the Department of Radiology, Stanford University School of Medicine, San Francisco, California, with the aid of a grant from the National Cancer Institute, U. S. Public Health Service, Federal Security Agency.

EXPERIMENTAL PROCEDURE

Four experimental groups of female F_1 hybrid mice of strain L × A parentage were established. Litter-mate pairs were assigned respectively to groups I and II, or to groups III and IV. Three variables: (*a*) irradiation of the host, (*b*) *in vivo* irradiation of ovarian grafts, and (*c*) ovariectomy, were manipulated in an attempt to evaluate possible direct and indirect, and local *versus* systemic, effects of irradiation on ovarian tumor induction. The experimental procedures that characterize each group are summarized in table 1.

TABLE 1.—*Incidence of ovarian and lymphoid tumors in spayed and intact LAF_1 female mice bearing irradiated and nonirradiated ovarian grafts*

Group	Whole-body irradiation	Bilateral ovariectomy	Intramuscular ovarian grafts	Net number of mice	Mice with tumors in ovarian grafts		Mice with lymphoid tumors	
					Number	Percent	Number	Percent
I	Yes	Yes	Nonirradiated	31	[a] 1	3	20	64
II	No	Yes	Irradiated	26	19	73	9	35
III	Yes	Yes	Irradiated	28	17	61	13	46
IV	No	No	Irradiated	30	0	0	14	47

[a] This tumor was a sarcoma that could not definitely be said to have originated in ovarian tissue.

Mice of group I received whole-body irradiation in a single dose of 300r at about 1 month of age.[3] Bilateral ovariectomy was performed 7–21 days later, at which time the freshly excised ovaries of a group II mouse, usually the original littermate, were implanted into the adductor muscles of each hind leg.

Group II mice similarly were ovariectomized and received intramuscular grafts of the ovaries of group I mice, but were not irradiated.

Animals of group III were exposed to a single whole-body dose of 300r at about 1 month of age.[3] Bilateral ovariectomy was performed 7–21 days later. One of the excised, preirradiated ovaries was autologously grafted into the adductor muscle of the right hind leg of each mouse. The other excised ovary was simultaneously implanted intramuscularly into the corresponding litter-mate animal of the intact, nonirradiated fourth group.

Thus, the experimental design presents several possibilities. The occurrence of tumors in the irradiated grafts of otherwise untreated hosts (group IV) would indicate clearly that a direct local effect of irradiation exists and is an adequate stimulus. The failure of tumors to appear in this group would not exclude a direct local effect, but would imply that such an effect, if it exists, is unable to elicit tumors in the absence of an indirect hormonal imbalance. A direct local effect could be dismissed only if tumors were to occur with significant frequency in the nonirradiated grafts

[3] Physical factors of irradiation were: 186 KVP, 20 ma., 0.25 mm. Cu and 0.55 mm. Al filter, focus-skin distance 47 cm., output 66 r/min.

of irradiated, spayed group I mice. Finally, an estimate of the influence of whole-body irradiation might be gained from the comparative results in groups II and III (both of which were composed of spayed hosts bearing irradiated grafts), since only the latter group was irradiated.

All animals received Purina laboratory chow and water *ad libitum*, and were maintained under identical laboratory conditions. Daily vaginal smears were made for several weeks in selected mice from all four groups. After an interval of about 4 months, vaginal smears of the same mice were again studied daily for approximately 2 weeks.

Animals were observed for the development of cysts and tumor masses in the ovarian transplants and were killed when they developed very large tumors or became moribund for other reasons. Autopsy was performed on all animals, with routine examination of the ovarian grafts, the uterus, adrenals, kidneys, liver, spleen, lungs, thymus, lymph nodes, and the intact ovaries in mice of group IV. The mammary glands and the vagina were also studied in a few mice. Histologic sections of all of these tissues were routinely prepared and examined, except where excessive autolysis had occurred.

OBSERVATIONS AND RESULTS

The incidence of ovarian tumors by groups is summarized in table 1. Irradiated grafts implanted into mice with intact ovaries (group IV) yielded no tumors. Irradiated ovaries implanted into ovariectomized mice, either irradiated (group III) or nonirradiated (group II), gave rise to a high incidence of tumors (61 and 73 percent, respectively). Only 1 (3 percent) of 31 irradiated ovariectomized mice bearing nonirradiated ovarian grafts developed a tumor at the site of the implant (group I). This was a sarcoma that could not definitely be said to have originated in ovarian tissue.

Table 2 indicates that most of the observed ovarian neoplasms were granulosa-cell tumors, similar to those described elsewhere (*8*, *9*, *13*, *17*). Metastasis to the liver was noted in one instance. There were also 4 luteomas, 6 tubular adenomas, and 1 thecoma; several other tumors exhibited mixtures of more than one tumor-cell type. Granulosa-cell tumors were more frequent among tumor-bearing mice of group II than among those of group III. Cysts of various sizes, filled with either clear or bloody fluid, were often found in the nontumorous grafts as well as in association with tumors.

Cyclic vaginal smears were observed in all animals so studied among the nontumor-bearing groups, I and IV; all but one of these again exhibited cyclic smears on the repeat study about 4 months later (table 2). In contrast, 15 of 18 mice in groups II and III showed persistent anestrous smears on the initial study. However, on subsequent re-examination, 7 of 9 mice in group II, and 4 of 9 in group III had developed either cyclic or persistently cornified vaginal smears. The return of cornified cells to the vaginal smears of animals bearing irradiated ovaries has been previously described by Geist, Gaines, and Escher (*19*).

TABLE 2.—*Ovarian tumors and vaginal smears in LAF_1 female mice bearing intramuscular ovarian grafts*

Group	Status of host	Status of graft	Number of grafts	Number of mice	Ovarian tumors [a]							Vaginal smears [b]					
					G	L	M	Th	Ta	S	None	Initial			Repeat		
												+	±	−	+	±	−
I	Spayed	Nonirradiated	2	31	0	0	0	0	0	1	30	10	0	0	10	0	0
II	Spayed	Irradiated	2	26	17	2	3	0	4	0	7	0	2	7	7	0	2
III	Spayed	Irradiated	1	28	6	2	4	1	2	0	[c] 11	1	0	8	4	2	3
IV	Intact	Irradiated	1	30	0	0	0	0	0	0	30	9	0	0	8	0	1

[a] G—Granulosa-cell tumor; L—luteoma; M—mixed-cell tumor; Th—thecoma; Ta—tubular adenoma; S—sarcoma.

[b] + = cyclic or persistently cornified; ± = occasional cornification; − = persistent anestrus.

[c] Two other tumors were autolyzed and not histologically classifiable.

The longevity of LAF_1 hybrid mice is well known (*20*). In the present experiment, the average survival times of the four groups were, respectively, 613, 707, 654, and 809 days. The relatively greater number of early lymphoid tumors in mice of group I was probably responsible for their somewhat shorter life span. Since animals were not killed until they became moribund, and most of the ovarian tumors grew very slowly, survival time was not a good index of the latent period for ovarian-tumor development following irradiation.

Lymphoid tumors were observed in many of the animals in all groups (table 1). However, the differences in incidence among groups were not statistically significant at the 0.05 level. The observed incidence ranged from 35 to 64 percent, with an average of 49 percent, which tallies very closely with the incidence previously observed by Lorenz and associates (*20*) in LAF_1 hybrid mice. In general, the histologic character of these lymphoid tumors appeared to fall into two main groups that were at least roughly correlated with the survival time. Lymphomas appearing relatively early tended to be lymphocytic or lymphoblastic, and were essentially similar to those previously observed in other strains of mice following whole-body irradiation (*4*). Most of the lymphoid tumors that occurred in mice surviving 600 to 800 days had a more pleomorphic character, with a predominance of larger, less deeply staining cells and less evidence of invasion. These have been designated by various observers as stem-cell lymphomas, reticulum-cell sarcomas, or clasmatocytomas. Detailed studies aimed at clarifying the histologic nature of these tumors were not made in the present experiment.

A miscellany of other tumors occurred sporadically in all groups. These included two adrenal medullary tumors (pheochromocytomas) similar to those recently reported by Smith, Gardner, Li, and Kaplan (*21*). Also noteworthy were an epidermoid carcinoma of the renal pelvis and an epidermoid carcinoma of the forestomach, the etiology of which was not apparent.

DISCUSSION

Irradiated ovaries grafted intramuscularly into untreated mice did not give rise to tumors, in contrast to the behavior of irradiated ovarian grafts in spayed hosts. This result is consistent with the observation of Lick, Kirschbaum, and Mixer (*18*) that the presence of an intact ovary inhibits the development of tumors in the locally irradiated opposite ovary. These findings appear to support the thesis that radiation-induced ovarian tumors of mice arise indirectly, presumably in response to hormonal imbalance resulting from radiation injury to the ovaries, with impairment of ovarian endocrine function. Such an interpretation is in keeping with available evidence concerning the mechanism of development of the histologically similar tumors that occur in intrasplenic ovarian grafts in gonadectomized mice (*15*).

However, the experimental observations reported here cannot properly be interpreted to exclude a direct local effect of irradiation in ovarian tumorigenesis. Such a conclusion would have been warranted only if a

number of tumors had been observed in mice of group I. All that can be said is that a direct local effect of irradiation, if it exists, is not an adequate stimulus for ovarian-tumor development in intact hosts (group IV) in which there is no reason to anticipate a pituitary-ovarian imbalance.

The concept of a direct local effect is not without some experimental support, albeit of inferential nature. Ovarian tumors have been observed in mice exposed to single x-ray doses of as little as 50r, as well as in animals chronically exposed to gamma radiation at a rate of only 0.1r per day (*20*). Animals exposed to such low doses exhibit cyclic vaginal smears and are capable of bearing normal litters with apparently normal frequency (*22*). There is no way, at present, of evaluating the sensitivity of the vaginal smear and of fertility as indices of ovarian endocrine function. Nevertheless, these are the best criteria currently available. Since ovarian tumors may be induced by doses of ionizing radiation so small as to effect no detectable disturbance of ovarian hormone secretion, a direct effect of radiation on ovarian tissue would appear to be implicated in the neoplastic process.

Evidence can therefore be adduced to support both an indirect hormonal mechanism and a direct local effect of irradiation in ovarian-tumor formation, under experimental conditions so designed as to minimize one factor and perhaps exaggerate the other. It seems likely, however, that under most conditions *both* factors would be effective, perhaps by synergism or summation, in leading to the development of ovarian tumors in irradiated mice.

Finally, brief comment should be made about the role of systemic irradiation. In unpublished incidental observations (*23*, Exp. 1), local irradiation yielded ovarian tumors only when the ovaries were included in the treatment field. This observation is supported by the work of Lick, Kirschbaum, and Mixer (*18*), and by certain of the results of the present experiment. The frequency with which mice of groups II (nonirradiated hosts) and III (irradiated hosts) developed ovarian tumors did not differ significantly. Thus, systemic irradiation does not appear to influence the incidence of these neoplasms. However, the relative frequency of granulosa-cell tumors was distinctly greater among mice of group II. It would be of interest to repeat this part of the experiment under more critical conditions, since this result carries the very interesting implication that systemic irradiation may influence the histology of induced ovarian neoplasms, possibly through some effect on the pituitary.

SUMMARY

Irradiated ovaries implanted intramuscularly into irradiated and nonirradiated groups of spayed LAF_1 hybrid mice, gave rise to many granulosa-cell tumors, luteomas, and related neoplasms. No such tumors occurred when irradiated ovaries were implanted into nonirradiated, nonovariectomized mice. Nonirradiated ovarian grafts in irradiated, spayed animals yielded only one sarcoma (3 percent) that could not definitely

be said to have originated in ovarian tissue. Cyclic vaginal smears were consistently observed in selected animals of the latter two groups, whereas anestrous smears occurred initially in 15 of 18 mice of the tumor-bearing groups, giving way within about 4 months to cyclic or persistently cornified smears in 11 of these. It is concluded that intact ovarian endocrine function inhibits the development of tumors in irradiated ovarian grafts, confirming the reported observations of Lick, Kirschbaum, and Mixer (*18*). The significance of these results is discussed in relation to other available evidence, and it is suggested that both a direct and an indirect mechanism are involved in the development of tumors in irradiated mouse ovaries.

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SURVIVAL, BODY WEIGHTS, AND LYMPHOID - TISSUE WEIGHTS FOLLOWING ADRENALECTOMY IN CBA MICE [1] [2] [3]

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INTRODUCTION

The use of adrenalectomized animals in all but very acute experiments requires the maintenance of these animals in good health and the demonstration of a sustained reduction of adrenal function following operation. While a considerable literature is available on dogs and rats (*cf. 1*), comparatively little attention has been devoted to this problem in mice (*2–6*). The present report describes the use of an easily prepared dietary regimen which has proved to be of value in some strains of mice. Evidence for a continued reduction in adrenal cortical function after adrenalectomy has been sought by observations on lymphoid-tissue hyperplasia and on survival following dietary manipulation.

METHODS

Young male mice of strain CBA (Strong) were raised on Purina laboratory chow until weaning at age 4 weeks. They were given additional rations of bread and milk for 1 week and operated upon when 5 weeks of age. Bilateral adrenalectomy was performed in one stage *via* the lumbar route. The procedure differed from that routinely employed for rats in only one essential respect. In contrast to the rat, the kidneys and adrenal glands of the mouse cannot be readily mobilized through the lumbar incisions. Excellent exposure has been obtained, however, by dissection of the anterior and medial attachments of the kidneys and lateral retraction of these organs. By employing sharp dissection, one may excise the adrenal glands and surrounding fat in one block. Ether and approximately one-third the anesthetizing dose of sodium pentobarbital have provided satisfactory anesthesia.

Immediately after operation, the mice were fed the bread and milk diet only. This was prepared by soaking commercial white bread in whole milk to which dried brewers' yeast (30 grams per liter) had been added. Several determinations indicated that the final proportions were surprisingly uniform—the average values being 1.1 kilograms of bread to 1.8

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[3] These studies were begun in the Biophysics Section of the National Cancer Institute and completed in the Department of Radiology, Stanford University School of Medicine, San Francisco, Calif.

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liters of milk. The percentage composition, calculated (7) on the basis of dry weight, is: protein 19, carbohydrate 68, fat 10, and ash 3. The drinking water of the operated animals contained 0.9 percent sodium chloride throughout. In addition, 5 percent glucose was added for the first 3 postoperative days (*8*)

SURVIVAL ON TEST REGIMEN

The bread and milk regimen supported 86 percent (60 of 70) of adrenalectomized CBA mice in these experiments. The majority of deaths (7 of 10) occurred within 48 hours of operation. This is in accord with the greater cortin requirement of adrenalectomized dogs in the early postoperative period (*9*); and may be related to the additional stresses attending surgical trauma (*10*). In view of the strain and colony variations found in rats, it is not surprising that different survival rates were observed for other strains of mice. In a group of 20 females of strain A and a group of 40 males of strain C57 Black,[5] mortality rates of 95 percent and 42 percent, respectively, were noted during a 2-week observation period following adrenalectomy.

Preliminary data showed that approximately 80 percent of adrenalectomized CBA mice failed to survive when the bread and milk regimen was replaced by Purina laboratory chow and tap water. More clear-cut results have been obtained by compelling the animals to drink a solution of 1 or 2 percent potassium chloride. In the first experiment, 2 groups of animals were prepared, a sham-operated group and an adrenalectomized group. After operation, the animals were maintained on the bread and milk regimen for a period of from 1 to 3 weeks. This was considered a sufficient interval to avoid confusion with deaths due in part to the surgical procedure. A test regimen was then instituted which consisted of Purina laboratory chow and 1-percent-potassium-chloride solution as drinking water. The results are recorded in table 1. Within 2 weeks, 20 of 21 adrenalectomized animals had died, while all of the control group survived. The drinking water was then changed to a 2-percent-potassium-

TABLE 1.—*Mortality of male CBA mice following application of test regimen* [1]

Experiment No.	Number of mice	Operation	Interval: operation to test	Number of mice [2] tested	Deaths during test
1	20	Sham	1–3 weeks	20	0
	23	Adrenalectomy	1–3 weeks	21	20
2	12	Sham	4–6 weeks	12	0
	16	Incomplete adrenalectomy	4–6 weeks	15	4
	19	Adrenalectomy	4–6 weeks	18	17

[1] Test regimen in Experiment 1 consisted of Purina laboratory chow and 1 percent potassium chloride as drinking water. In Experiment 2, the same diet and 2 percent-potassium-chloride drinking water were employed. Animals were raised on bread and milk diet with 0.9 percent sodium chloride as drinking water in the interval between operation and test.

[2] The difference between this figure and the original number of mice indicates those dying in the interval between operation and test.

[5] These mice were from stocks maintained at the National Cancer Institute, Bethesda, Md.

chloride solution. The single surviving adrenalectomized animal died 9 days later, while the control group was maintained without loss for an observation period of 8 weeks.

In a sense, this first experiment establishes the validity of the test regimen in distinguishing between animals with intact adrenal function and those made acutely adrenopryvic. A more critical experiment would involve application of the test after a sufficient interval to allow for hypertrophy of accessory adrenal bodies or regeneration of adrenal cortical tissue from remnants inadvertently left behind at surgery. For this purpose, 3 groups of animals were established. A sham-operated and an adrenalectomized group were prepared as in the previous experiment. In a third group, the operation was intentionally incomplete; the entire right adrenal gland was excised, while a portion (15 to 25 percent) of the left adrenal gland was left behind at surgery. The test regimen of Purina laboratory chow and 2-percent-potassium-chloride drinking water was instituted after a postoperative interval of from 4 to 6 weeks. No mortality was encountered in the sham-operated group; 4 of 15 incompletely adrenalectomized animals and 17 of 18 completely adrenalectomized animals died during the test (table 1).

Adrenal tissue was not found at autopsy in 3 of the 4 incompletely adrenalectomized animals that died following application of the test. These examinations were made difficult by post-mortem tissue changes. The surviving animals of this group showed considerable variation in the extent of the regeneration that took place in the adrenal remnants. In general, however, the volume of the regenerated fragment appeared to be less than one-half that of an intact gland. Autoptic examination of the surviving animal of the completely adrenalectomized group did not reveal any adrenal cortical tissue.

BODY WEIGHTS AND LYMPHOID-TISSUE-ORGAN WEIGHTS

This experiment provides data for a comparison of the growth rates of intact and adrenalectomized animals. At the completion of this portion of the study, the organ weights of various lymphoid tissues were also determined. It was thought that the hyperplasia of lymphoid tissues following adrenalectomy is so well established that these organ weights would provide an additional indication of the level of adrenal cortical activity.

Three groups of animals were prepared. One of 2 sham-operated groups was fed Purina laboratory chow and tap water, while the other received the bread and milk diet and tap water. An adrenalectomized group was maintained on the latter diet and saline drinking water. Both diets were fed *ad libitum*. Individual body weights were determined weekly on a double beam balance. When the animals were aged 12 weeks (7 weeks after operation), they were killed by cervical dislocation. Autopsies were performed in a routine manner, which included a general examination and an inspection of the operative site for residual adrenal tissue or evidence of postoperative infection. The following organs were dissected free of

surrounding tissue and weighed on a precision torsion balance: the thymus gland, the spleen, the adrenal glands, and a sample of the peripheral lymph nodes, which included the 2 axillary nodes on both sides and the 2 epigastric nodes. Statistical analysis involved the application of the "t-test" (*11*) and significance was attached to differences wherein a p-value=<0.01 was obtained.

The data are summarized in table 2. The body-weight data were so similar for the 3 groups that only the weights at weaning, at 8 weeks, and at autopsy are recorded. All animals showed a progressive weight gain that was not interrupted by the operation, and significant differences were not observed between any of the 3 groups. The growth of the adrenalectomized animals appeared quite comparable with that of either control (sham) group. Organ-weight data reveal excellent agreement between the values for the 2 control groups. All the lymphoid tissues examined were of significantly greater weight in the adrenalectomized group than in either of the other two groups.

The data were then examined for the possibility that they might provide an indication of the level of adrenal cortical activity of an *individual* animal. When the spleen weights were referred to an arbitrary value of 110 mg., it was found that the spleens of 2 sham-operated animals were heavier, and the spleen of 1 adrenalectomized animal was lower than this value, yielding a total "error" of 5 percent. Similarly, an "error" of 11 percent was obtained when either thymus-gland- or lymph-node weights were employed. Thus, the hyperplasia of lymphoid tissues may provide a rough index of reduced adrenal cortical activity.

DISCUSSION

Quantitative evaluation of adrenal cortical function following bilateral adrenalectomy is extremely difficult if not impossible. A return of such function after operation is theoretically possible on the basis of (1) regeneration of cortical tissue from glandular capsule inadvertently left behind at surgery, (2) hypertrophy of accessory glandular bodies not removed along with the gland itself, and (3) differentiation of "adrenal cortical-like" tissue from coelomic mesothelium or ovarian tissue (*12, 13*). Furthermore, Speirs (*6*) has recently discussed evidence which suggests that the endocrine function of regenerated adrenal cortical tissue may be sufficient to support life, though inadequate for certain physiological responses, especially those associated with stress. It is thus clear that considerable caution is indicated before accepting an adrenalectomized animal as a preparation *totally* lacking in adrenal cortical function.

Of the present experiments, it may be said that careful surgical technique will provide a preparation in which there is a physiologically demonstrable reduction in adrenal cortical function for at least 7 weeks after operation. The magnitude of this reduction cannot be accurately appraised, chiefly because of the difficulty in measuring the stress imposed by the test regimen and thereby evaluating the level of adrenal cortical function required to survive it. The high survival rate of the incompletely adrenal-

TABLE 2.—*Body weights and lymphoid-tissue-organ weights of male CBA mice*

[Operations performed and experimental diets begun at 5 weeks of age: autopsies performed at 12 weeks of age]

Group No.	Number of mice	Operation	Diet	Body weight (gm.) at age			Organ weights (mg.)			
				4 weeks	8 weeks	12 weeks	Spleen	Lymph nodes	Thymus	Adrenal glands [1]
1	17	Sham	Purina laboratory chow	16.9 ±.5	24.7 ±.5	27.9 ±.3	80 ±3.4	26 ±.8	36 ±1.3	3.3 (2–5)
2	19	Sham	Bread and milk	16.6 ±.7	26.4 ±.5	29.3 ±.5	90 ±5.1	27 ±1.6	32 ±1.1	3.3 (2–4)
3	21	Adrenalectomy	Bread and milk	18.1 ±.5	26.0 ±.5	28.7 ±.4	134 ±4.2	39 ±1.4	50 ±1.3	----------

[1] Values are means and range of extreme values. Other values are means and standard error by $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$

ectomized group and the relatively small volume of their regenerated cortical tissue can be considered as evidence that the stress imposed by the test regimen did not require high levels of cortical function for survival. On the other hand, the fact that a few of these animals failed to survive the test can be interpreted in the converse manner, but must be evaluated in the light of the possibility that surgical trauma interfered with the vascular supply of the adrenal remnant and regeneration failed to occur. The similarity of the results of the test when applied immediately, and at a considerable interval, after operation would likewise indicate that a significant contribution to the level of adrenal cortical function was not made by such sources as the coelomic mesothelium.

It is felt that the sum of the evidence justifies the statement that the adrenalectomized preparation described in this report is a suitable one for studies in which it is desired that adaptive changes in adrenal cortical function be reduced to a minimum for periods up to 2 months. The evidence that such studies may be carried out without the complication of malnutrition is of considerable practical, as well as theoretical, importance. Although absolute growth standards cannot be obtained for an individual strain of mice, both the dietary constituents employed and the growth rates observed compare favorably with proposed standards (*14*).

Finally, comment may be made on the observations of Stoerk (*15*) that the thymus-gland weights of adrenalectomized rats, while greater than those of intact animals on the same diet (Rockland pellets), were not greater than those of intact animals on a different and presumably more adequate diet (diet E). He has suggested that many laboratory animals respond to inadequate nutrition by "accidental involution" of the thymus gland, and that adrenalectomy simply abolishes this response. In the absence of absolute standards, the adequacy of the bread and milk diet employed in the present experiments cannot be proved beyond question. As noted above, however, the growth curves recorded herein compare well with proposed standards and give no suggestion of malnutrition. It is, therefore, difficult to explain the observed hyperplasia of the lymphoid tissues following adrenalectomy as merely the abolition of an involutional response to inanition.

SUMMARY

A dietary regimen is described that adequately maintained a large proportion of adrenalectomized mice for periods up to 7 weeks after operation. A simple test regimen distinguished between sham-operated and adrenalectomized individuals, and between completely and incompletely adrenalectomized groups. The inference was drawn that the adrenalectomized mice remained at least relatively adrenopryvic (without the ability to alter greatly their adrenal cortical function) for several weeks after operation.

The growth curves of the adrenalectomized mice compared favorably with those of sham-operated controls and with those proposed as standards for mice on complete diets. Seven weeks after operation, the lymphoid organs of these mice were significantly heavier than those of their controls.

It was concluded that the hyperplasia of lymphoid tissues following adrenalectomy is not dependent upon the abolition of an involutional response to malnutrition.

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THE OCCURRENCE OF TUMORS OF THE FORESTOMACH IN MICE AFTER PARENTERAL ADMINISTRATION OF METHYLCHOLANTHRENE: A HISTOPATHOLOGIC AND GENETIC ANALYSIS [1] [2] [3] [4]

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Epidermoid carcinomas of the forestomach occur occasionally in untreated mice (*1*, *2*) and more frequently in experimental mice. Some carcinogenic substances applied directly to the stomach or administered with the diet have been associated with the tumors produced experimentally. An analysis of forestomach tumors occurring after a single subcutaneous injection of 20-methylcholanthrene is presented in this study.

Numerous methods for the administration of a carcinogen have been employed by other investigators. These include: gavage with a carcinogenic solution (*3*), feeding of carcinogenic compounds (*4*), suspending oily solutions of the carcinogen in drinking water (*5*, *6*), the topical application of the compound to a somatic area accessible to the tongue of the animal (*3*), and the injection of the carcinogen directly into the wall of the stomach (*7*, *8*, *9*). In all but the last experiments the tumors have originated either in the forestomach or at the limiting ridge, and have predominantly manifested the classical histologic picture of epidermoid carcinoma. The tumors resulting from intramural injection of a carcinogen included adenocarcinoma, adenoacanthoma, and sarcoma, as well as epidermoid carcinoma.

Many tumors observed in the present study were histologically identical to the epidermoid carcinomas described in the papers cited above. A second group of tumors resembled spindle-cell sarcomas which have been described as occurring at the site of the subcutaneous injection of methylcholanthrene (*10*, *11*) or at the site of the injection of methylcholanthrene directly into the wall of the stomach (*9*). The third group contained both spindle cells and epidermoid cells. Tumors of this nature also have been reported as occurring at the site of injection of a carcinogen (*10*, *11*). A

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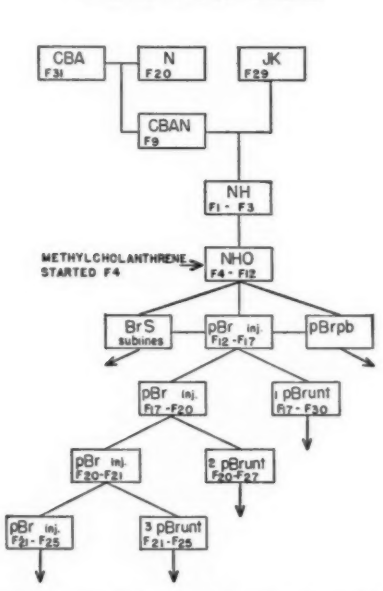
fourth group of lesions was seen. These consisted of papillomas similar to those observed after several types of treatment (*3, 6, 12*) and were regarded as benign.

MATERIALS AND METHODS

Although the general procedure employed in this laboratory has been described (*13*), a brief review with special reference to the subline studied in this investigation is indicated. The gastric neoplasia occurred in the pBr (pink eye, brown) subline. Its relation to other sublines of the NHO strain is illustrated in text-figure 1.



TEXT-FIGURE 1.—The origin of the NHO strain and the sublines considered in this paper. Arrows indicate that the sublines are being continued.

At 60 days of age each mouse of the NHO strain and hence the pBr subline received a subcutaneous injection of 1 mg. of methylcholanthrene dissolved in 0.1 cc. of sesame oil. In nearly all instances brother to sister matings from the fourth to the twenty-fifth generation of the NHO strain served to maintain the genetic homogeneity of the animals. Except for interruption of treatment with methylcholanthrene at the generations indicated in text-figure 1, the pBrunt subline should be identical genetically with the pBr.

The mice were observed weekly in order to detect the appearance of local tumors at the site of injection or of tumors at other sites, and to observe the general physical condition of the mice. They were autopsied either at death or when it was apparent that death was imminent. At

autopsy the skin, extremities, and viscera were examined. All the tumors from animals in which the stomach was involved in neoplasia were fixed in Bouin's solution, sectioned, and stained with hematoxylin and eosin. If, for example, a mouse had a gastric lesion and a pulmonary or skin tumor, these were also studied. In many instances Masson, Laidlaw's silver reticulum, and Van Gieson's stains were employed to aid in the final classification of the tumors of the forestomach.

It was necessary to eliminate 8 percent of the first 2,500 mice injected with methylcholanthrene for one of the following reasons: (1) escape; (2) death, followed by decomposition beyond which an adequate autopsy could be performed; (3) death of the animal before the first observation 2 months following the administration of the carcinogen; or (4) destruction of the carcass by the cage mates. The mice were maintained on a diet of Nurishmix supplemented once per week with whole mixed grains. Water was supplied *ad libitum*. The animals were kept in an air conditioned room with the temperature constant at 74° F. and the humidity between 50 and 60 percent.

The NHO strain has been established and maintained by inbreeding animals that were most resistant to cancer following the injection of methylcholanthrene (*13*). Animals from earlier generations had tumors at an earlier age than those of later generations. Inasmuch as the stomach tumors were characterized by relatively long latent periods, it was necessary that some degree of resistance to local tumors be established in order to allow individuals to live long enough to develop the lesions of the stomach. The gastric tumors were studied with reference to, *a*, their morphology, *b*, heredity of the hosts, *c*, age of appearance, *d*, incidence, and *e*, transplantability.

OBSERVATIONS

Morphology

Gross examination usually revealed a nodular surfaced, pink or white translucent mass that had replaced all or part of the forestomach and at times the whole organ (fig. 1 *A*, *B*). The tumors were firm and appeared to originate on the anterior surface of the stomach adjacent to the lumen of the esophagus (fig. 1 *C*, *D*). Some of them contained abscesses from several millimeters to a centimeter in diameter. Others contained focal areas of hemorrhage. The larger tumors ranged between 2 and 3 cm. in diameter. Masses of neoplastic tissue extended into the spleen, liver, pancreas, esophagus, and diaphragm, and occasionally into the body wall and erector spinae muscles. Metastases were often seen in the liver, diaphragm, pancreas and mesenteries, and occasionally in the kidney, lung, and genital tract. The brain was not examined routinely. Clues related to the histologic types of the tumors were not discernible by gross examination.

Forty-five of the tumors were considered to be epidermoid carcinomas. The neoplastic-cell type was nearly identical in all of these tumors, variation among members being manifest by differences in stromal content,

degree of necrosis, number of mitoses, and to a lesser extent, degree of maturation of the neoplastic cells. These cells were arranged in broad sheets and strands, seldom displaying intercellular spaces (fig. 2 *A*). Prickle cells were sometimes observed. The nuclei were generally hyperchromatic, ellipsoid, or round, one diameter never exceeding 2 or 3 times the other. The prominent nuclear membrane was characterized by a serrated internal surface. The chromatin was dispersed as deeply stained particles or strands. One or two prominent nucleoli were usually seen in each cell (fig. 2 *B*). Most of these tumors involved structures adjacent to the stomach. Spread of the tumor was apparently manifest by the following sequence. The neoplastic cells originated on the anterior surface of the forestomach near the lumen of the esophagus. It was not clear whether the neoplasia necessarily occurred at the base of a papilloma. Invasion of the muscularis followed with extension through the muscular layers and the serosa as well as extension along the muscularis to other areas of the forestomach and into the muscularis of the pylorus. Early involvement of the mesentery adjacent to the stomach was observed. Apparently this tissue served as an avenue of extension for the tumor into the spleen, liver, pancreas, and mesenteric lymph nodes. Isolated tumorous nodules in the lungs, liver, and kidneys were seen in animals bearing advanced tumors (figs. 3 *A*, *B* and 4 *A*). Several well defined instances of tumor thrombi were observed within the lumina of veins.

Some tumors contained many acinus-like structures (fig. 4 *B*). Necrosis of cells lying at the center of a tumor nodule, farthest from their blood supply, was regarded as the major factor contributing to this phenomenon. The nuclei appeared to be displaced to one side of the cell by a deeply acidophilic, spherical globule of the cytoplasm (fig. 4 *C*, *a*). These droplets, frequently about twice the size of the nuclei, gradually lost their eosinophilia (fig. 4 *C*, *b*). Vacuoles were observed and interpreted as the next step in this process (fig. 4 *C*, *b*). Pyknosis and death of the cells followed, leaving spaces surrounded by surviving cells (fig. 4 *C*, *c*). Some of these acinus-like spaces contained debris. In two cases this material had a definite structure. Hyaline eosinophilic spheres ranging in size from about 1 to 6 μ were found lying free within the acinus-like structures. Occasionally these globules were encircled by a regular, thick walled, highly refractile membrane (fig. 4 *B*).

Nineteen of the tumors were classified as spindle-cell neoplasms. The largest neoplastic mass in each tumor replaced the muscularis of the forestomach, and projected into the lumen (fig. 5 *A*). The cells of the epithelium covering these tumors were hyperplastic, hyperchromatic, and hyperkeratotic. Ulceration of the epithelium occurred frequently. These tumors were usually organized as interlacing bands of tumor cells oriented in all planes. The cells were spindle shaped with oval nuclei (fig. *5 B* and 6 *A*). The cytoplasm was sparse and pale blue with hematoxylin and eosin stain. The cell membrane could not be distinguished clearly. In general the long axes of the nuclei were 2 to 5 times the length of the short axes. Variation in size and shape was considerable. A well developed

nuclear membrane encircled a moderate amount of chromatin, which was dispersed in small granules and intercommunicating strands. Single and multiple, prominent nucleoli occurred frequently. Although distant metastases were less frequent than in mice with epidermoid tumors, invasion of adjacent organs was a prominent feature (fig. 5 *B*, *C*).

It is necessary to emphasize that variation in cell type and cellular arrangement within this group of tumors was considerably broader than that within the group of epidermoid carcinomas. The term spindle-cell neoplasm was chosen as descriptive of the cell type most frequently observed. Nevertheless, round cells and pyramidal cells were numerous. Although differences between the extremes were obvious, differences between intermediate individuals of this group were so minute that they were classified together. A large amount of reticulum was demonstrable in these tumors. It was distributed more or less homogeneously as a fine network within which the tumor cells appeared to be suspended (fig. 6 *B*). Reticulum fibers were notably scarce in portions of tumor that were not contaminated by invaded tissue; for example, in areas that bulged into the peritoneal cavity or grew between layers of mesentery.

Seventeen of the tumors were classified as mixed tumors. These contained both epidermoid and spindle cells. The cytology of the respective parts of the tumors was in general identical to that described in each of the two preceding sections. It was impossible to estimate the relative proportions of the two types, inasmuch as serial sections were not available. It is likely that there was no constant relationship, some tumors being composed of nearly all spindle cells with a small number of epidermoid cells while in others the opposite relationship was apparent. The reticulum distribution characteristic of the epidermoid carcinomas and also of the spindle-cell neoplasms was represented in the mixed tumors as well as areas of transition between the two extremes (fig. 6 *C*). The transition between the two types in many instances was so gradual that sharp definition of the two areas was impossible (fig. 7 *A*, *B*, *C*, *D*).

Thirty-one papillomas of the forestomach were observed. These occurred either singly or at 2 or 3 independent loci (fig. 8 *A*). They were characterized by a thickening and an outfolding of the squamous epithelium, and hyperkeratosis (fig. 8 *B*). Some were pedunculated, but the majority were sessile. Some tumors in the group of epidermoid carcinomas were approximately the same size as these benign papillomas but extension of neoplastic cells through the lamina propria and muscularis had occurred. Extension of epithelial elements along small vessels at the base of some papillomas was apparent.

Three animals contained adenomatous lesions of the pylorus as well as papillomas of the forestomach. The pyloric lesions consisted of large cystic structures that were lined by well organized columnar epithelium. These structures penetrated the lamina propria glandularis to appear as multiloculated cysts within the muscular layers of the pylorus (fig. 8 *C*). The lesions were identical to those described as the lesion characteristic of the Brs subline (*13–16*).

The single adenocarcinoma observed in this series was about 6 mm. in diameter and was situated on the greater curvature of the stomach at the limiting ridge. The proximal margin of the lesion was continuous with the squamous epithelium of the forestomach and the distal margin with the columnar epithelium of the body of the stomach (fig. 9 *A*). The luminal surface of the tumor was ulcerated and somewhat infiltrated by polymorphonuclear cells, lymphocytes, and macrophages. The neoplastic cells were cuboidal and contained large round nuclei and basophilic cytoplasm (fig. 9 *B*). Mitoses were abundant, and atypical forms were observed. The cells were arranged as acini throughout most of the tumor except in areas in which the muscularis was being infiltrated. All muscularis layers were invaded by cancer cells, and the tumor appeared to have penetrated the serosa to emerge free within the peritoneal cavity. No metastases were observed. This mouse was classified with the group that contained spindle-cell neoplasms because an independent spindle-cell neoplasm occurred in the forestomach.

Two of the gastric tumors observed could not be classified. One of these (from mouse 206212) was located primarily in the muscular wall of the pylorus and the body of the stomach, distal to the limiting ridge. It had widely invaded the glandular mucosa, and was composed of a single uniform cell type (fig. 10 *A*). These cells were round, and contained homogeneously blue-staining cytoplasm (hematoxylin and eosin stain) and an eccentrically placed round nucleus (fig. 10 *B*). The chromatin was distributed in large masses radiating from the center of the nuclei. The cells were similar to plasma cells. A tumor that occurred spontaneously in a mouse and from its description was nearly identical to this has been reported (*1*). The other tumor was similar to those classified as adenoacanthomas. It was composed of cords of cuboidal and flat cells that apparently originated from the squamous epithelium of the forestomach. Many of the cells were organized into acini. The size and shape of the deeply basophilic, round nuclei were remarkably constant. The glandular mucosa, pancreas, and mesenteric lymph nodes were widely invaded. The neoplastic extensions into these organs more closely resembled glandular tissue.

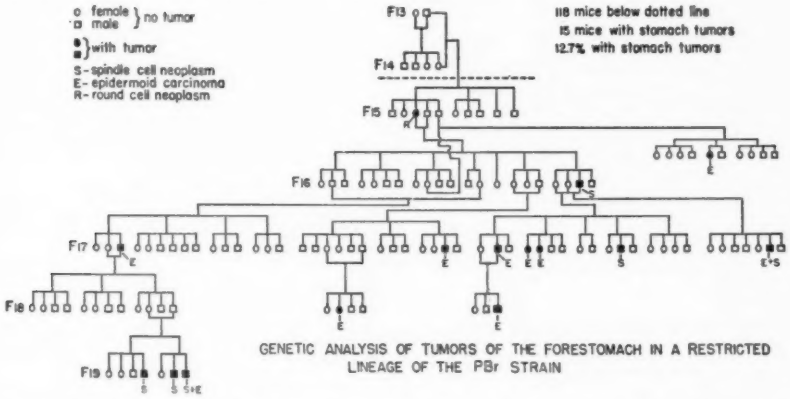
Other tumors: Thirty-seven animals had pulmonary tumors as well as stomach tumors. Tumors of this nature occur spontaneously in mice or occur after a variety of experimental procedures. They have been described as possible bronchial adenomas and recently as pulmonary adenomas (*17*, *18*). Sharply defined and well oriented basophilic columnar or cuboidal cells formed well organized acini. The spherically shaped tumors rarely infiltrated the parenchyma of the lung. Mitoses were rare. In addition, the lungs of several mice contained metastases from gastric tumors.

Five mice had local tumors at the site of injection of the carcinogen. These tumors were compatible with those previously described as occurring at the injection site in mice of this strain (*11*).

GENETIC RELATIONSHIPS

An intimate familial relationship among tumor-bearing animals was found in one lineage within the pBr subline. One hundred and fifteen mice descended from mouse 206212 over a period of 5 generations. Within this group 15 stomach tumors were observed (text-fig. 2).

The incidence was 12.7 percent as opposed to an incidence of about 3 percent among all animals of the pBr descent studied. Thus, 18.5 percent of the gastric tumors was observed in a single family. This family, however, included only about 4 percent of the total number of experimental animals. The morphology of the gastric tumors in this high tumor family was not constant, all types of lesions being represented. The round-cell tumor

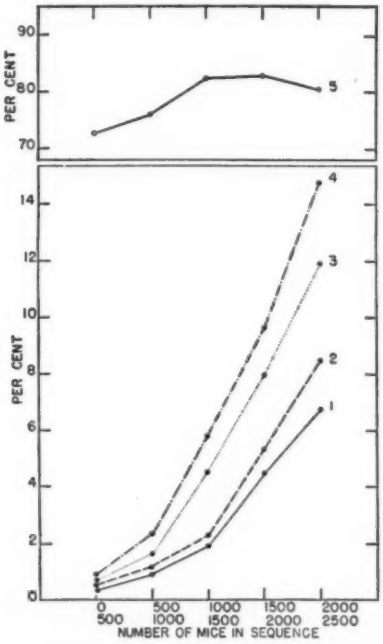


TEXT-FIGURE 2.—Chart depicting the genetic relationships among the descendants of mouse 206212.

described above was found in the mouse from which the individuals in the high-tumor family were descended. In addition, 20 of the gastric tumors occurred in siblings.

INCIDENCE OF FORESTOMACH TUMORS

The incidence of gastric tumors was evaluated by plotting the percent of mice with tumors in successive groups of 500 animals (text-fig. 3). For example, 0.43 percent of the mice in the first 500 mice in the experiment acquired gastric tumors (curve 1); 0.89 percent in the second 500, *etc.* The other curves represent the percentages of gastric tumors in various subdivisions of the groups of 500 mice. Curve 2 indicates the percentage of mice with gastric tumors among mice with tumors of all types. Curve 3 represents the percentage of mice with gastric tumors among those mice that lived longer than 250 days, the minimal latent period observed for gastric tumors. Curve 4 shows the percentage of gastric tumors in those mice that lived longer than 250 days and that also had tumors of all types. Curve 5 illustrates the percentage of tumors



TEXT-FIGURE 3.—Incidence of gastric tumors and the incidence of tumors of all types observed in the first 2500 mice of the pBr subline. See text for explanation of the graph.

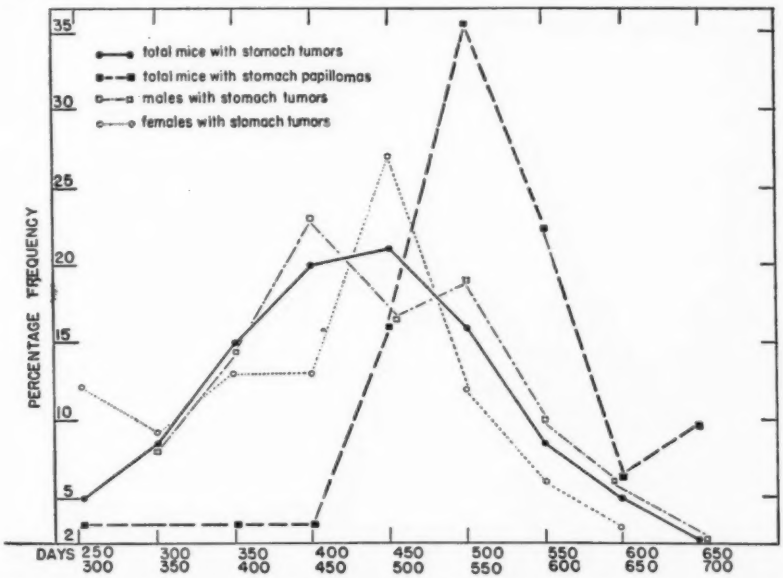
of all types. The increase in incidence of gastric tumors from the early to the late generations included in the experiment closely paralleled the increase in incidence of gastric tumors in the successive groups of 500 mice.

LATENT PERIODS

The latent period of a tumor was defined as the number of days from the injection of methylcholanthrene to the autopsy of the mouse, minus 30 days. A percentage frequency curve of the latent periods of the gastric tumors revealed a distribution compatible with that of a normal distribution curve (text-fig. 4). The individual points in the curves were connected by straight lines in order to facilitate clarity and brevity rather than to present the data as a series of histograms. A slight but statistically insignificant difference between the latent periods of the males and females was observed. While it is not shown directly in text-figure 4, differences between the latent periods of the three histologic types of malignant tumors were also insignificant. A difference of 75 days between the mean latent period of the malignant forestomach tumors and the mean latent period of the epidermoid papillomas of the forestomach was observed. Inspection of the percentage frequency curves elucidates the relationships between the malignant and benign lesions of the forestomach.

TRANSPLANTATION

Five epidermoid carcinomas were transplanted into mice of the same subline. All tumors grew in spite of the fact that some were septic at the time the original transplant was carried out. Four of these transplantable tumors were carried for several generations and then discontinued. The fifth, taken from mouse 245931, displayed some unusual characteristics and is now in its eighteenth transplant generation. The original tumor and some of the tumor transplants are illustrated (fig. 11 *A*, *B*, *C*, *D*). In the early transplant generations this tumor was lethal to the hosts within 30 to 60 days following transplantation. In the succeeding transplant generations the lethal properties became more pronounced. At present the hosts die from 10 to 30 days following transplantation. The cause of death in most instances has not been determined. Although widespread metastases from the subcutaneous transplants have occasionally been seen, these did not seem responsible for the death of the host in many instances. A profound change in the morphology of the neoplastic cells occurred in the third transplant generation of this tumor. The fragments of tumor were placed into the peritoneal cavity of the new hosts. Within 2 weeks some of the hosts died. Hemorrhage into the peritoneal cavity or intestinal obstruction was the cause of death. Histologic examination of the transplants revealed that the neoplastic cells had changed from the epidermoid to the spindle-cell type. In fact, the tumor transplants could not be distin-



TEXT-FIGURE 4.—A percentage frequency curve of the latent periods of the malignant and benign tumors of the forestomach. Each point represents the percent of mice in a given class with latent periods falling between the days represented by the abscissa. The classes are indicated in the figure.

guished from the forestomach tumors classified as spindle-cell neoplasm by means of any of the staining methods employed.

DISCUSSION

Although only 84 tumors of the forestomach occurred in about 3,000 treated mice, analysis of the characteristics of these tumors as well as comparison with other groups of mice in the laboratory, both treated and untreated, indicated a direct relationship between the subcutaneous injection of methylcholanthrene and the occurrence of the tumors. The small percentage of forestomach tumors is less distressing when viewed with respect to the over-all incidence of internal tumors that occurred in this subline. The increase in percentage of forestomach tumors with succeeding generations was a reflection of the increase in incidence of a larger group of tumors that included all neoplasms occurring at a site remote from that of the injection of the carcinogen.[7] The increase in tumors of the forestomach, in spite of genetic selection designed to increase the resistance of the subline to cancer, emphasizes the role of methylcholanthrene as a potent factor in the genesis of the lesions. The expression of an unknown recessive gene or a mutation due to factors other than those related to the administration of methylcholanthrene seemed unlikely.

Epidermoid carcinomas of the forestomach produced by methylcholanthrene added to the drinking water and identical to some tumors in this series required nearly one hundred times as much carcinogen (*5*). The latent periods were shorter, and the incidence was higher. The small amount of carcinogen possible for a mouse to ingest by licking his fur at the site of injection seems unlikely to be responsible for the lesions observed in this experiment. In another study, sarcomas and adenocarcinomas as well as epidermoid carcinomas resulted from the injection of methylcholanthrene into the wall of the stomach (*8*). The results following the subcutaneous injection of a carcinogen presented here, qualitatively, at least, parallel the latter study. This comparison seems compatible with the contention that the carcinogenic property of the methylcholanthrene was mediated *via* the parenteral route of administration.

Consideration of another subline of the NHO strain indicated that a genetic factor was concerned with the production of the gastric neoplasms. Text-figure 1 illustrates that the mice of the Brs subline had the same original genetic background as those of the pBr subline. The single observable difference between mice of the two sublines was eye color. The pBr mice had pink eyes, whereas the Brs had black. Both sublines have been subjected to identical experimental conditions. The mice of the Brs subline developed lesions of the pylorus (*14–17*). Only three lesions of this type have been observed in mice of the pBr subline. Rarely has an epidermoid carcinoma of the forestomach been seen in the Brs subline. This difference suggests that factors contributing to the control

[7] Strong, L. C., and Sanghvi, L. D. Genetic studies of chemically induced tumors in mice. Unpublished monograph.

of the susceptibility of the mouse to gastric neoplasms are genetically determined.

The occurrence of 18.5 percent of the tumors of the forestomach in a single family over a period of five generations and the occurrence of 25 percent of the tumors in siblings lend credence to the hypothesis that a genetic factor contributed to the production of the lesions. Inasmuch as there was no intentional selection toward the production of a family with a high-tumor incidence, this particular lineage was unrecognized until after it had been discontinued. Recognition of the high-tumor incidence in this family may have led to the establishment of a subline that would develop tumors spontaneously, as was the case with the pyloric lesion in the Brs subline (*13*).

The difficulty with which the spindle-cell areas could be demarcated from the epidermoid-cell areas in some of the mixed tumors suggested the possibility that, although histologically distinct, both types of tumor were fundamentally the same neoplasm. Qualities indicating that the two types belonged to separate populations were not demonstrable. Consideration of the sex distribution, heredity of the hosts, latent periods, incidence, and the properties of some of the tumor transplants indicated that the neoplasms were identical. While not proving that the two types of tumor were basically the same neoplasm, the evidence cited suggests that insistence of their independent neoplastic entities is impossible. The difficulties encountered in distinguishing sarcoma from anaplastic carcinoma have long been a problem in tumor classification (*19-23*). The significant factor to be considered in this discussion seems to be the observation that the origin of all the tumors of the gastrointestinal tract was limited to the stomach rather than a consideration of the exact nature of the lesions.

The observation that in general the latent periods of the benign papillomas were longer than the latent periods of the true neoplasms suggests that the papillomas were not necessarily precancerous lesions. Nutritional deficiencies in some of the older animals may have been responsible for some of the papillomas (*12*). The possibility that the tumors observed were preceded by papillomas has not been eliminated.

The factors that contributed to the development of the single adenocarcinoma in this series are obscure. The possibility that an adenocarcinoma of the stomach could be produced by the subcutaneous injection of a carcinogen is certainly conceivable, but the reasons why the lesion appeared in only one mouse are vague. It seems necessary to turn to the equally obscure question of biologic variability, which in this case must have been minimal, or to assume that other unknown extrinsic factors combined with the controlled conditions to produce this lesion.

The greater susceptibility of the forestomach to neoplastic change also suggests the existence of an extrinsic factor in the genesis of the experimentally-induced gastric tumors. The forestomach may not benefit as much as the glandular stomach from the gastric mucous barrier (*24, 25*). It seems reasonable at this time to hypothesize a mechanism of carcino-

genesis in these animals that combines several existing theories. First, a susceptible tissue is necessary (*26*). This may be prepared by treating a normal animal with a carcinogenic substance such as methylcholanthrene. Whether the susceptibility is induced by a change in the genetic constitution of the cells of the stomach or by a direct action upon the cells is not clear, but the former seems indicated by this experiment (*27*). With the stage set for carcinogenesis, an external or dietary factor may be necessary actually to produce the neoplastic change. The limitation of neoplasia of the gastrointestinal tract to the stomach suggests that the ingested form of the proposed dietary factor may not contribute to carcinogenesis. The exposure of this substance to the chemical action of the gastric secretion may be necessary to unmask its carcinogenic properties. The comparative lack of protection by the gastric mucous barrier would allow the forestomach to become the site of the neoplastic change.

SUMMARY AND CONCLUSIONS

A histopathologic and genetic analysis of 84 malignant tumors of the forestomach produced in mice by the subcutaneous injection of 0.1 mg. of 20-methylcholanthrene is presented. Forty-five of the tumors were epidermoid carcinomas, 19 were spindle-cell neoplasms, and 17 were mixed tumors.

In addition, 2 tumors of the stomach were seen that could not be classified. One of these was a round-cell tumor; the other seemed compatible with the diagnosis of adenoacanthoma. Other lesions of the stomach included 1 adenocarcinoma, 3 adenomatous lesions of the pylorus, and benign epidermoid papillomas which were observed in 31 mice. Pulmonary adenomas as well as stomach tumors were observed in 37 mice and tumors at the site of injection of the carcinogen in 5 animals.

The hereditary background of the mice in which the tumors occurred strongly suggested that a genetic factor was important as one of the etiologic principles.

No significant differences in the number of days required for a mouse to develop gastric tumors were observed among the classes of mice in which the malignant gastric tumors were found. A significantly longer time than that required for the development of the malignant lesions seemed necessary for the appearance of the benign papillomas.

It was impossible to conclude that the spindle-cell neoplasms were sarcomas rather than metaplastic forms of epidermoid carcinomas.

A mechanism for carcinogenesis in these mice is suggested.

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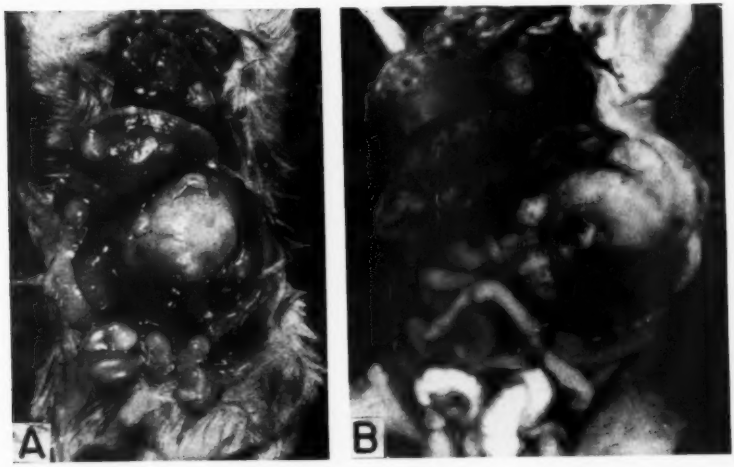
PLATE 4

FIGURE 1.—*A*, The appearance at autopsy of a representative carcinoma of the forestomach. Note the two large spherical metastases in the liver and the metastases in the genital omentum. The nodule in the left lung is an adenoma. *B*, A massive carcinoma of the forestomach involving adjacent organs (specimen fixed in 10 percent formalin). *C*, A carcinoma of the forestomach, with extension into the glandularis. Note the dilated esophagus. × 5. *D*, A spindle-cell neoplasm of the forestomach, with extension into the pancreas and adjacent mesentery. × 5.



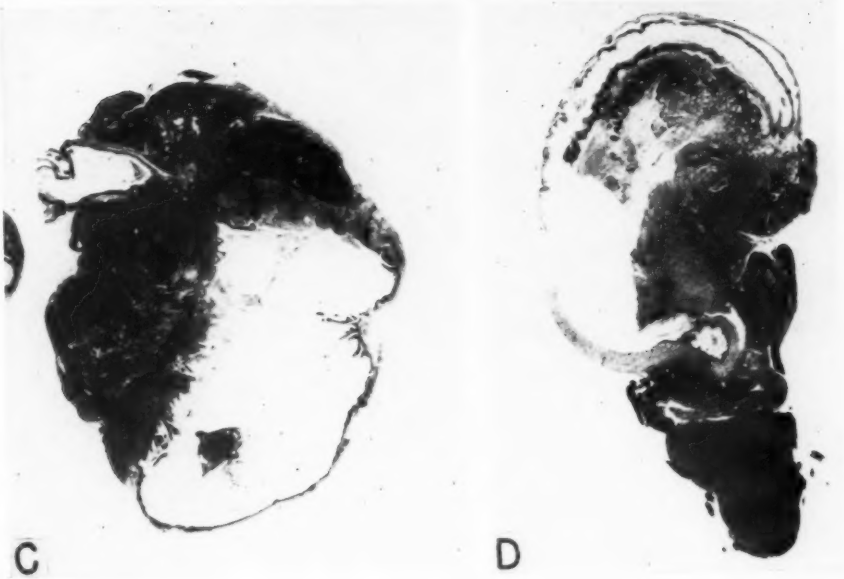


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PLATE 5

FIGURE 2.—*A*, Carcinoma of the forestomach. The luminal surface is on the labeled side of the illustration. × 40. *B*, Carcinoma of the forestomach. About × 280.

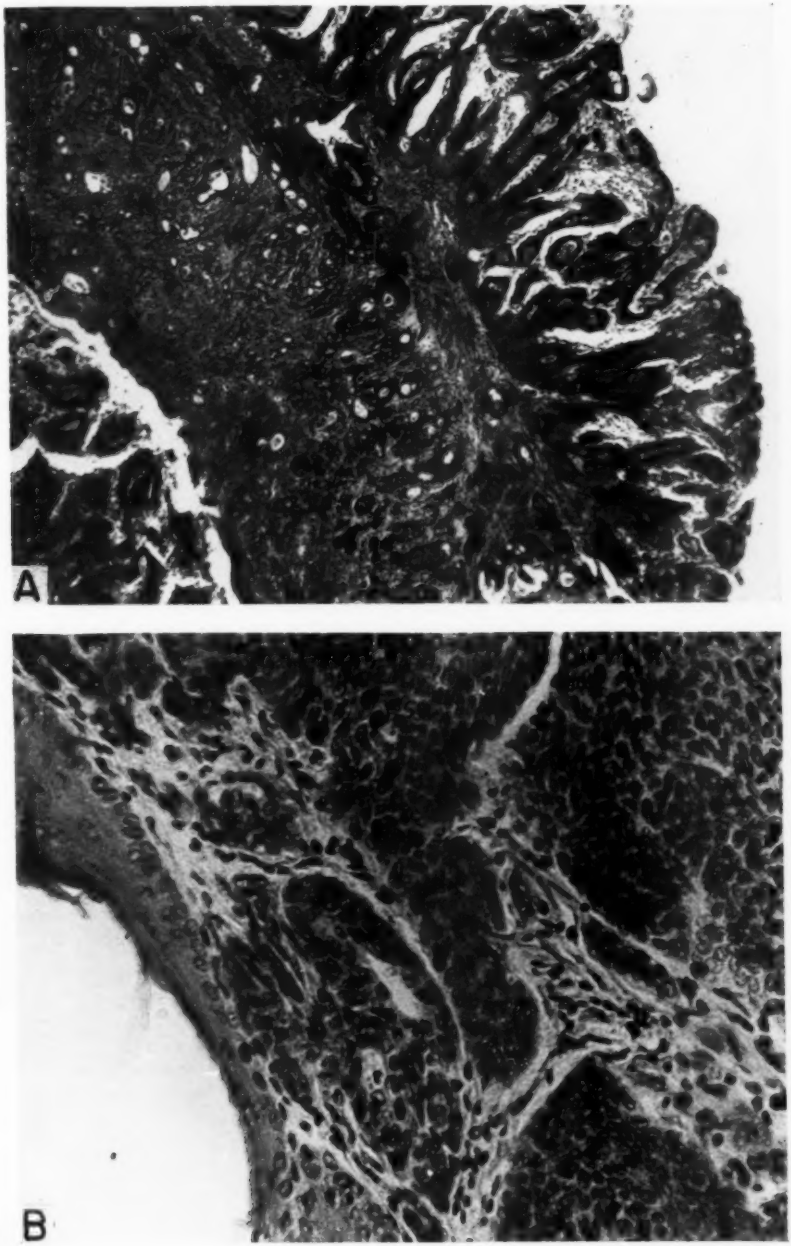
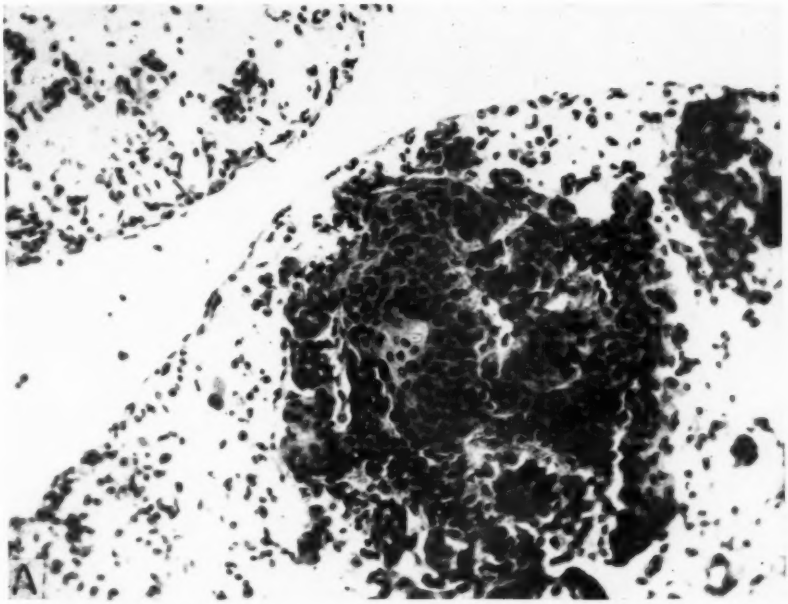

A
B

PLATE 6

FIGURE 3.—*A*, A metastases in the lung from an epidermoid carcinoma of the forestomach. About × 240. *B*, A thrombus of epidermoid carcinoma within an interlobular portal vein. Notice the mitotic figures. About × 280.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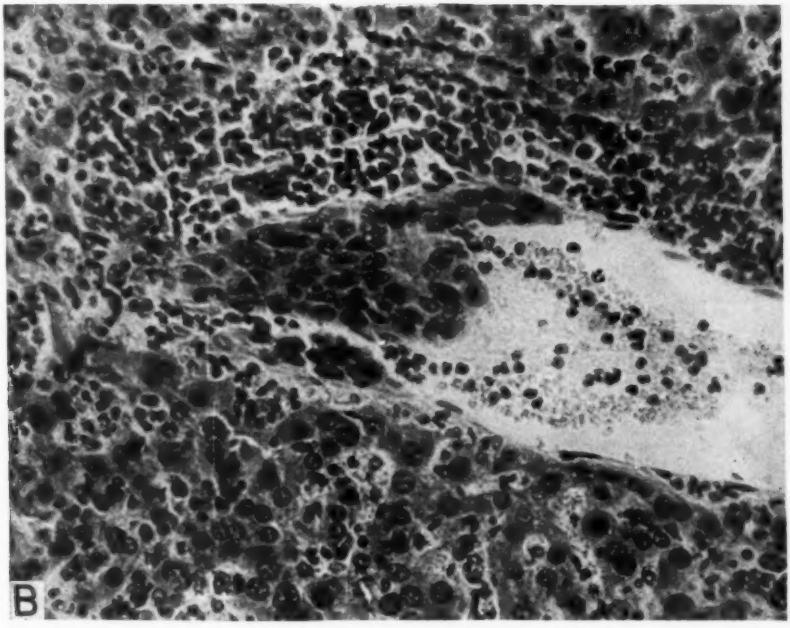
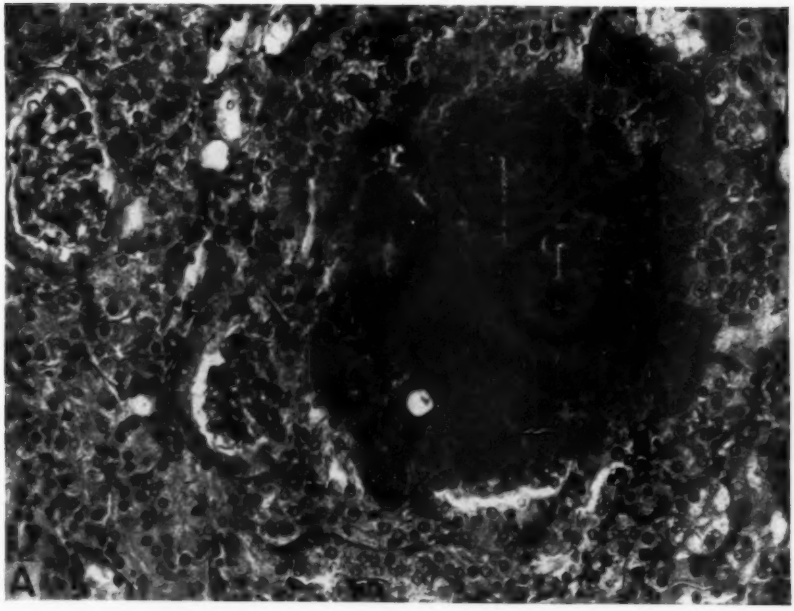

B

PLATE 7

FIGURE 4.—*A*, A renal metastases from an epidermoid carcinoma of the forestomach (244252). About × 240. *B*, Acinus-like structure of a forestomach carcinoma. Note the globular structure of material within an acinus, and the group of globular bodies encircled by a dense membrane (224725). About × 180. *C*, An epidermoid carcinoma of the forestomach illustrating the cytoplasmic changes that preceded the formation of the acinus-like structures. About × 240.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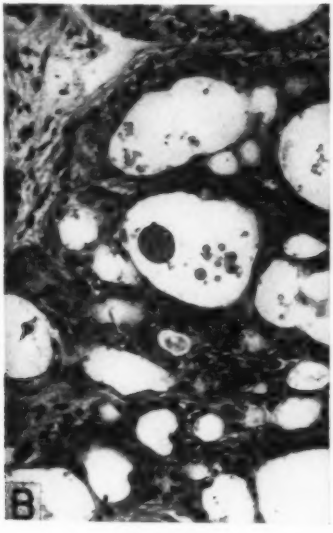
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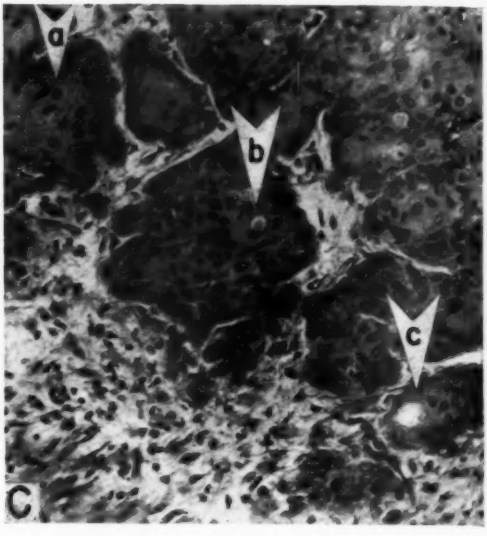
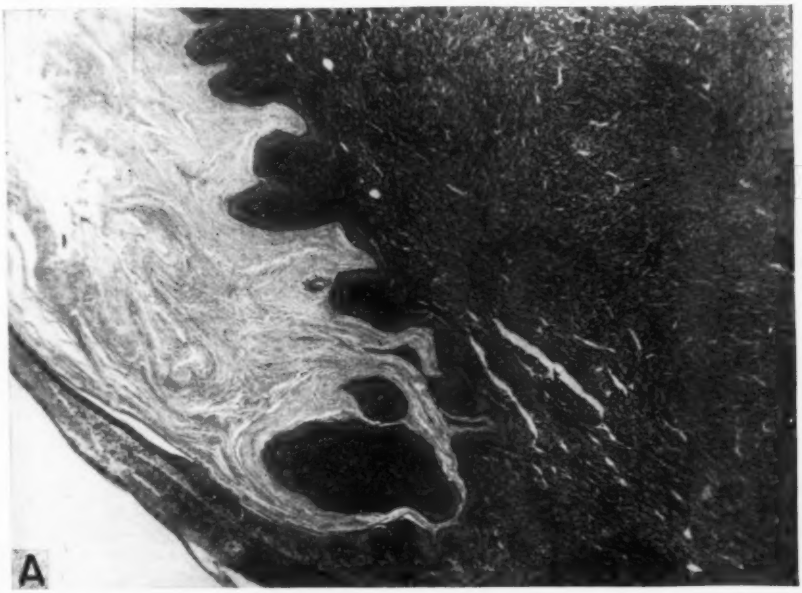

a
b
c
C

PLATE 8

FIGURE 5.—*A*, A spindle-cell neoplasm of the forestomach. Note the hyperplastic and hyperkeratotic epithelium covering the tumor, also notice the vascular spaces within the tumor (190845). About × 50. *B*, A spindle-cell neoplasm extending into the pancreas (225640). About × 240. *C*, A spindle-cell neoplasm invading the diaphragm (190845). About × 120.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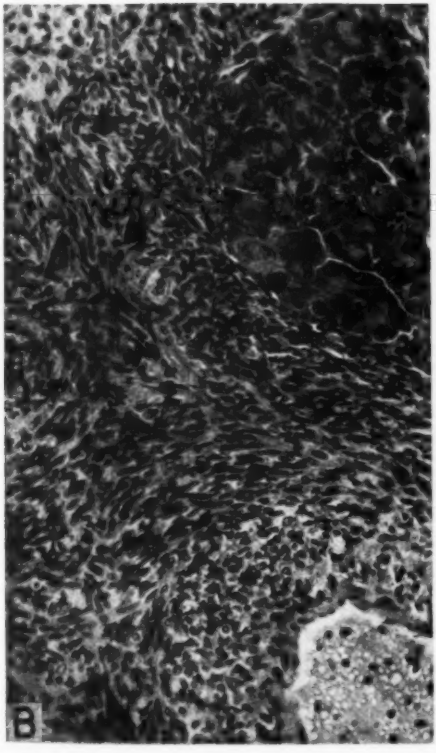
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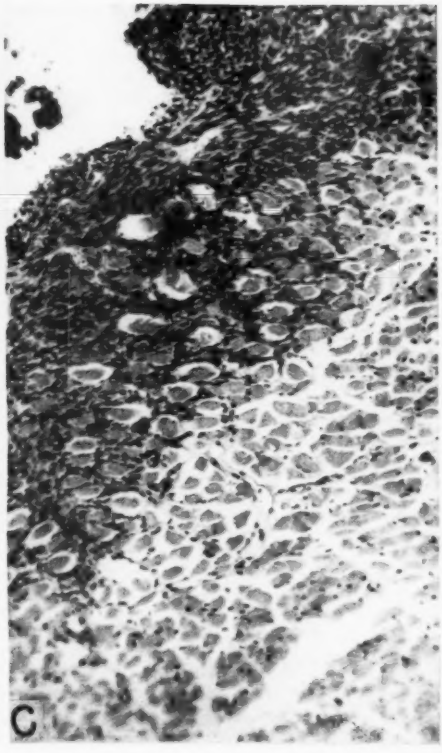
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PLATE 9

FIGURE 6.—*A*, A spindle-cell neoplasm of the forestomach. Despite the large size of the tumor it seems contained within the serosa in this area (116923). *B*, The reticulum distribution characteristic of most spindle-cell neoplasms (Laidlaw's stain for reticulum). × 120. Compare with *C*. *C*, The reticulum distribution of a mixed tumor at an area of transition (Laidlaw's stain for reticulum). The increase in the amount of reticulum apparently gradually divided the sheet of epidermoid cells into smaller segments. × 240.

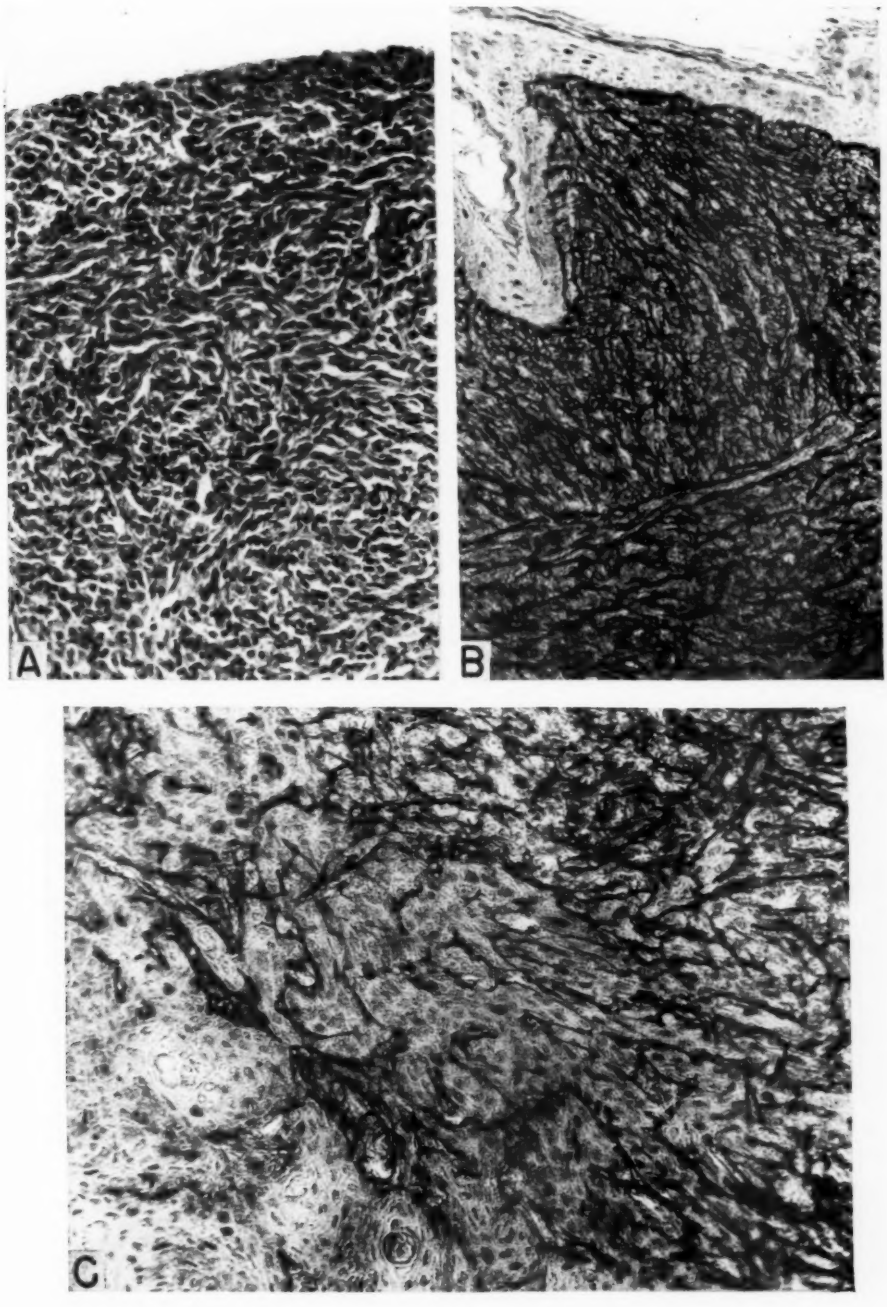
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PLATE 10

FIGURE 7.—*A*, *B*, and *C*, A mixed tumor. *A*, Prickle cells seen in the sheet of epidermoid cells illustrated in *B*. × 1200. *B*, An area of transition. About × 180. *C*, A spindle cell area. About × 180. *D*, A transitional area from another mixed tumor. About × 180.

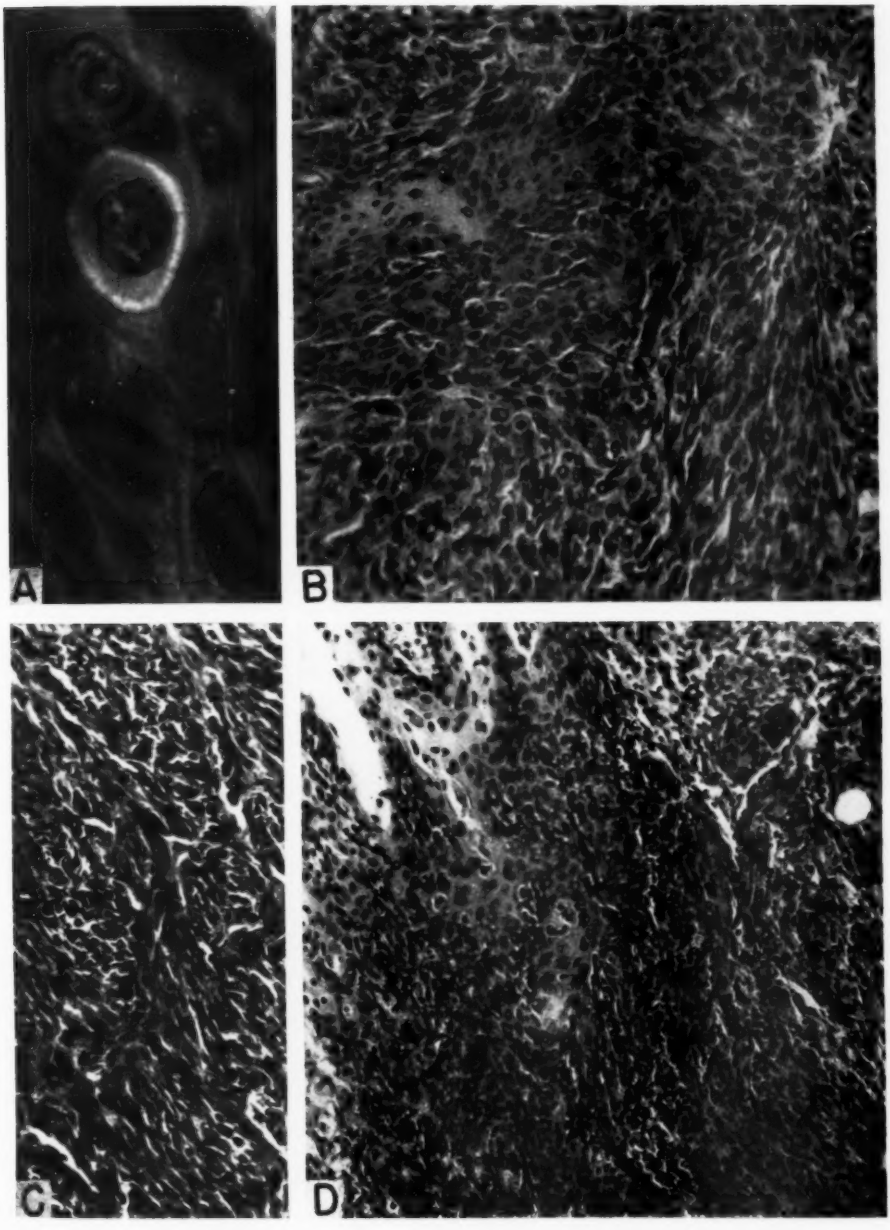
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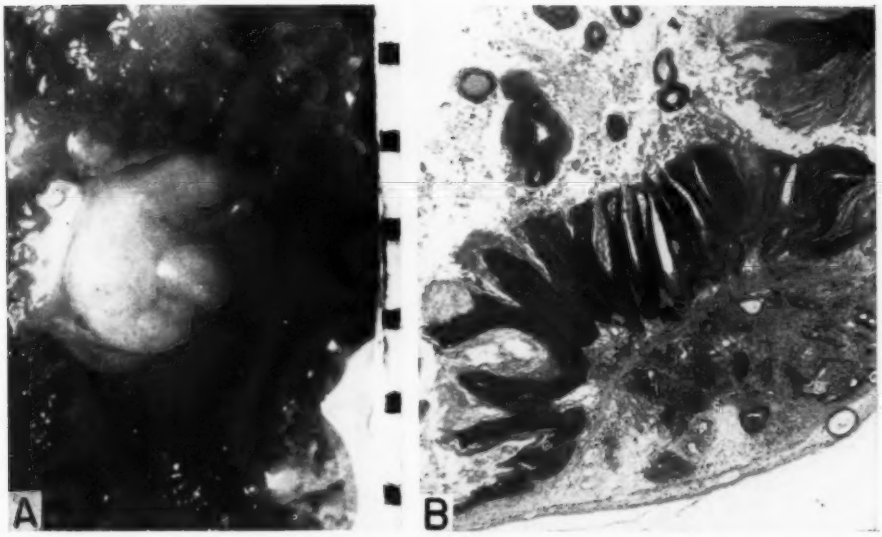
PLATE 11

FIGURE 8.—*A*, A gross illustration of one of the smallest papillomas found. The scale is in mm. *B*, An epidermoid papilloma of the forestomach. The cystic structures are lined by a single or double layer of squamous cells. × 40. *C*, An adenomatous lesion of the pylorus. The lesions extended deeply into the muscularis. The normal glands and the lamina propria glandularis are on the labeled side of the plate. × 120. Compare this lesion with that illustrated in fig. 9 *A*, *B*.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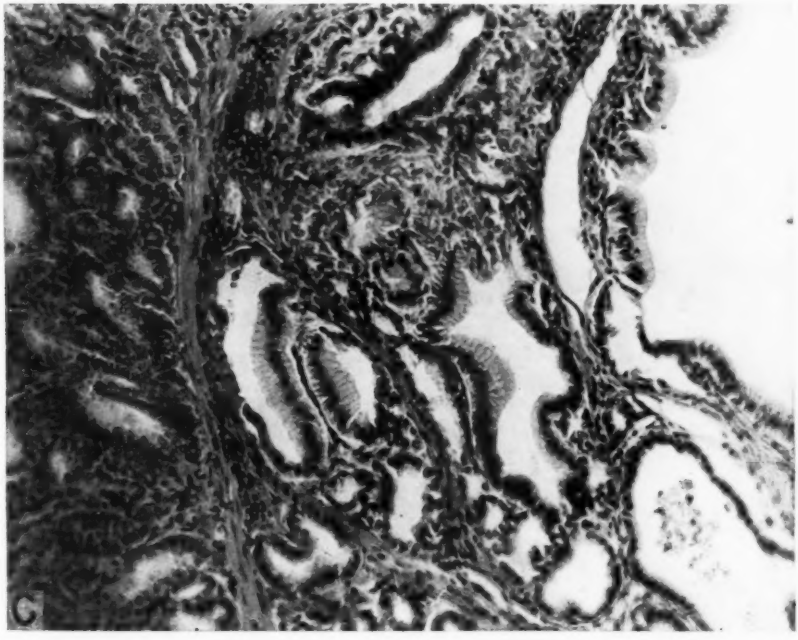
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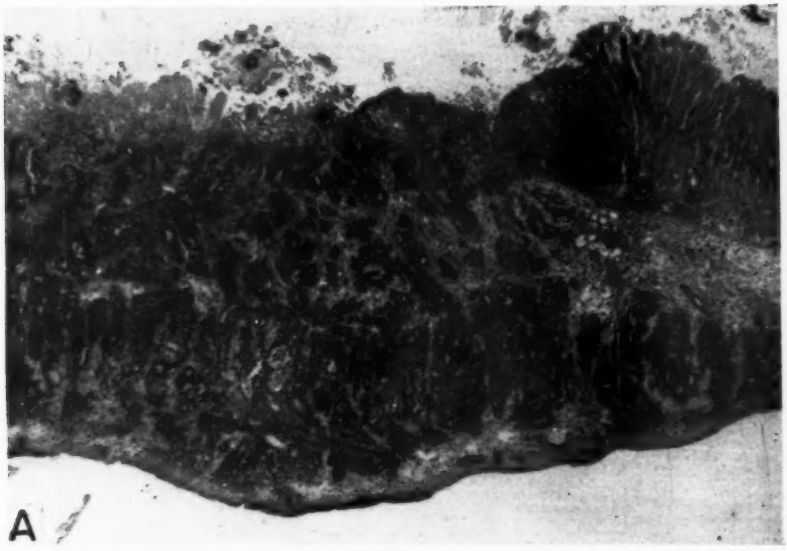
PLATE 12

FIGURE 9.—*A*, An adenocarcinoma of the stomach. × 40. *B*, The same lesion. About × 240.



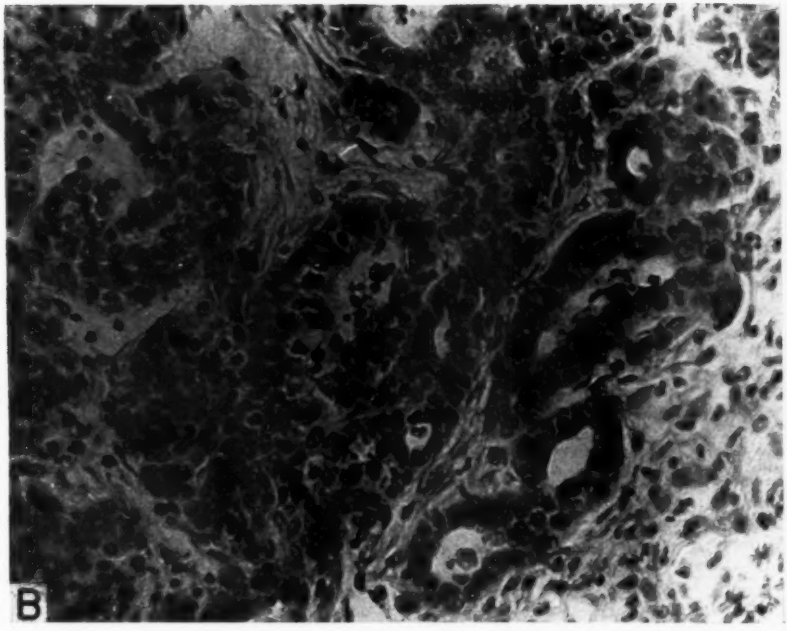


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PLATE 13

FIGURE 10.—*A*, A round cell tumor infiltrating the glandular mucosa of the stomach. About × 120. *B*, The same tumor infiltrating the pancreas, (206212). About × 240.

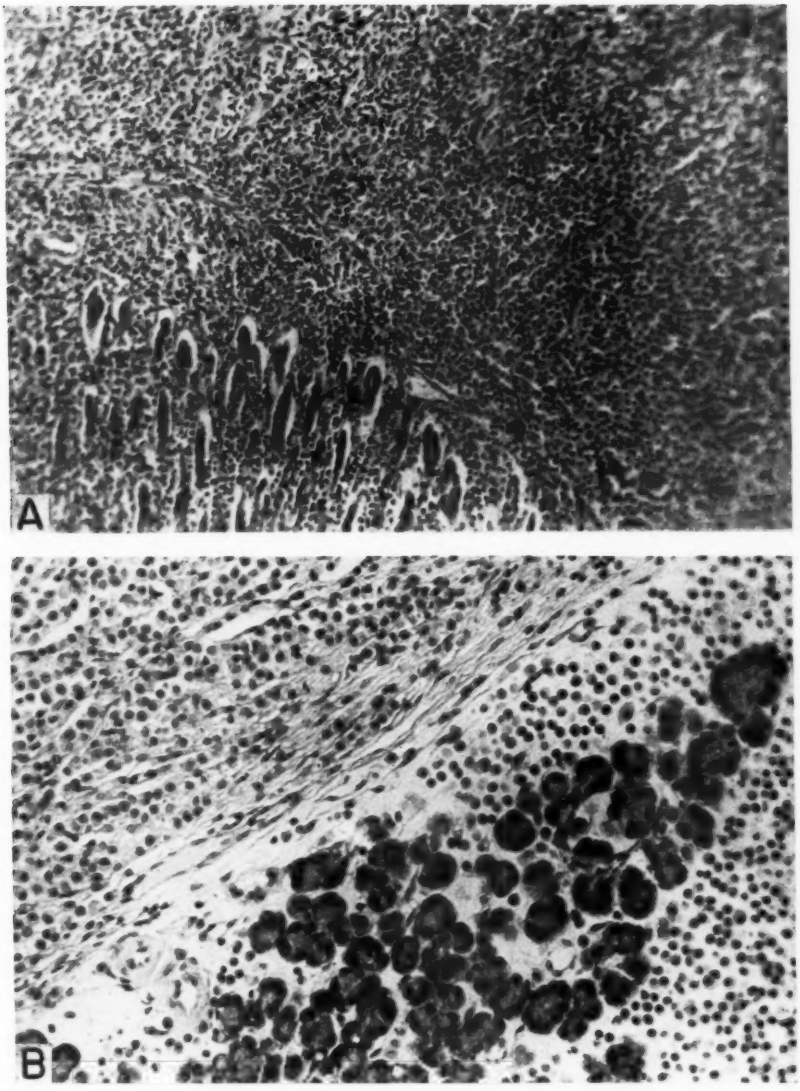
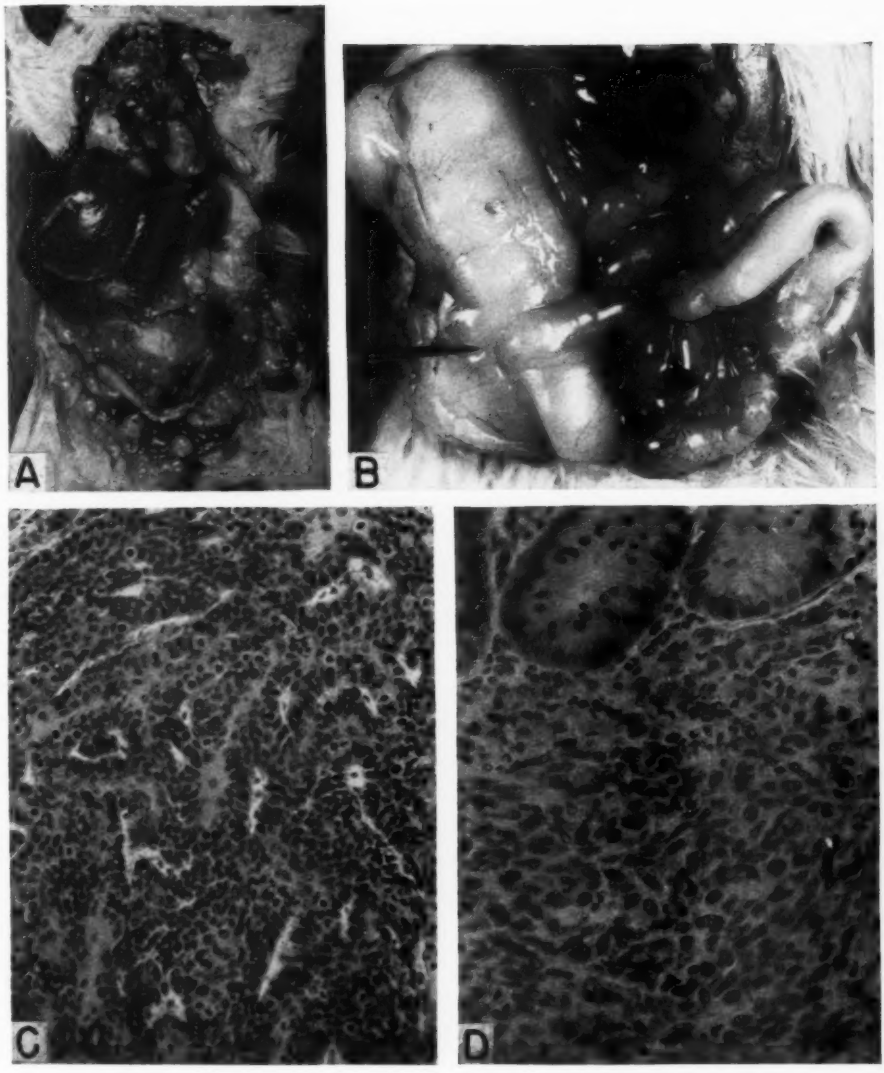

A
B

PLATE 14

FIGURE 11.—*A*, Widespread internal metastases which occurred in a mouse following the subcutaneous transplantation of a forestomach tumor (245931). Note the large intraperitoneal mass and also the mass in the left lung. *B*, A different subcutaneous transplant from the same tumor strain. The tumor has invaded the serosa of the small bowel causing obstruction. *C*, The original tumor from which the transplants A, B, and D were derived (245931). About × 100. *D*, An intraperitoneal transplant illustrating the change in morphology of the tumor cells. Compare with *C*. About × 100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REPORT OF TWO CASES OF IDENTICAL PRIMARY TUMORS INVOLVING SPINAL NERVE ROOTS AND MENINGES IN STRAIN NHO MICE [1]

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Secondary neoplastic invasion of the spinal canal and compression of the spinal cord have been observed occasionally in mice as a consequence of infiltration by leukemic cells. Less frequently spontaneous, induced, or transplanted tumors in the neighborhood of the vertebral column may extend into the canal. Primary tumors, however, arising from structures within the canal must be exceedingly rare. Therefore, a report of two such primary tumors in the same inbred strain of mouse is made. These are the first such tumors to be observed in the material examined clinically and pathologically at the National Cancer Institute comprising more than 70,000 autopsy accessions of mice. The Index to Literature of Experimental Cancer Research, 1930 to 1935 (*1*), has no report of tumors of this type. Scherer states that no spontaneous tumors of the central nervous system have been described up to the time of his publication in 1944 (*2*). Our colleagues, who have had a large experience with neoplasms in the mouse, have been queried and none of them could recall having seen a similar tumor.

REPORT OF CASES

Case 1. F–2799: An untreated strain NHO BrS brown female mouse, 15 months of age, showed weakness of the left foreleg and left hind leg. These were dragged when the mouse was urged to move forward. On direct stimulation an active thrust reflex was elicited. Voluntary motion was evident but not frequent or vigorous. The mouse made no spontaneous progressive forward motion but moved from side to side slowly. Two types of respiration were observed: a predominant, very rapid, shallow, diaphragmatic breathing, interspersed with an occasional slower more measured type of rhythm. The animal was killed by ether anesthesia.

Combined anatomic and histologic diagnoses: tumor of cervical spinal canal involving the cord, meninges, spinal nerves, spinal roots, and spinal ganglia with extension into the left brachial plexus and focal infiltration of the medulla oblongata, pons, and midbrain; simple cyst of ovary, bilateral; congestion and hemorrhage of mesenteric lymph node; Klossiella muris infestation of kidney; chronic hypertrophic gastritis; hyperkeratosis

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and papilloma of forestomach; and distention of urinary bladder with minimal cystitis.

The spinal cord was exposed by excising the laminae of the vertebrae and incising the dura, following a posterior midline incision and retraction of the muscles of the back. Lying within the arachnoid was an ovoid gray tumor, 1.8 by 1.5 by 1 mm., attached by a stalk-like structure to the left ventrolateral aspect of the cervical spinal cord (figs. 1 and 2). Dissection of the stalk revealed that it was composed of spinal roots. After removal of the spinal cord together with the local tumor mass the dura of the anterior wall of the spinal canal overlying the 5th, 6th, and 7th cervical vertebrae showed a depressed translucent, red-brown, smooth area, 4 by 1.5 mm., where the tumor had rested against the bony wall of the spinal canal. Transverse sections of the posterior body wall through this depressed area revealed a red gray nodular lesion extending along the brachial plexus in the soft tissues beneath the left scapula. Following removal of the cord, the attachment of the tumor to the cord was accidentally severed.

The tissues were fixed in acid Zenker's solution or in dilute solution of formaldehyde (U. S. P. 1–10). They were blocked in paraffin, cut and stained by the following methods: hematoxylin-eosin, Masson's trichrome, Mallory's aniline blue, van Gieson's connective-tissue, Weigert's elastic-tissue, Prussian blue reaction, phosphotungstic acid-hematoxylin, periodic acid Schiff reaction, Krajian's silver method for melanin, Wilder's silver method for reticulum, and Bodian method for nerve fibers.

A section through the spinal cord and local tumor mass revealed that the latter consisted of a spinal ganglion and its roots, both extensively permeated by tumor (fig. 3). The cord was compressed and distorted; however, only a narrow margin of the cord adjacent to the tumor showed neoplastic infiltration.

Sections of the cervical cord, medulla oblongata, pons, and mid-brain revealed a thin sheet of tumor cells spreading in the leptomeninges, especially along the ventral surface of these structures, with sporadic focal infiltration of tumor cells into the parenchyma by way of the Virchow-Robin spaces (figs. 4, 8, 9, and 10). One cranial nerve lying ventral to the hippocampus and considered to be the oculomotor (3d) nerve was infiltrated by tumor.

Sections through the bony wall of the cervical spinal canal revealed spinal ganglia and spinal and peripheral nerves infiltrated by tumor bilaterally, much less, however, on the right. The neoplastic cells permeated also the left spinal nerve in the corresponding intervertebral foramen (fig. 5) and the brachial plexus lying between the scapula and the posterior thoracic cage; the right spinal nerve was also involved but to a considerably lesser degree (figs. 6 and 7). Within the spinal canal there were atrophy and resorption of adjacent bone resulting from pressure of the neoplastic tissue, but osseous infiltration by tumor was not observed. Both inner and outer surfaces of the dura of the left anterolateral portion of the canal were covered by a thick layer of tumor, and the dura itself

was heavily infiltrated. The tumor infiltration diminished as the midline was approached and extended to only a limited degree to the right side.

Several nerve trunks comprising the left brachial plexus were considerably enlarged. The degree of involvement of individual nerve bundles varied, some being so heavily infiltrated by tumor cells that they were hardly recognizable as such. The epineurium was only slightly infiltrated. The surrounding fat and muscle ventral to the scapula showed evidence of compression but only minimal neoplastic infiltration; there was one large area of hemorrhage and necrosis beneath the left scapula.

No evidence of tumor was observed in sections through the thoracic and lumbar portions of the spinal canal, including surrounding tissues, muscle, fat, nerves, and ganglia.

The tumor cells were generally elongated, sometimes round or oval, averaging 25 microns in diameter, and showing only moderate variation in size, shape, and staining (figs. 11 and 12). The cytoplasm was fairly abundant, homogeneous, and slightly basophilic. The nucleus was almost always single, deeply hyperchromatic, and moderately variable in shape, being round, oval, lobated, or rod shaped. It had a heavily stained nuclear membrane and usually several heavily stained chromatin particles. An occasional cell had a large lobated nucleus, and rarely two or three nuclei were present in a single cell. Mitotic figures were infrequent, averaging not more than one per three or four high-power fields. Blood vessels were few in number, thin walled, and of a capillary type. There was relatively little inflammatory reaction, necrosis, or hemorrhage except for the area beneath the left scapula already referred to.

The nerve cells of the spinal ganglion permeated by tumor were rather sparse and showed no evidence of neoplasia. Some of the ganglion cells choked by tumor cells were degenerated and necrotic. Most of the nerve fibers of the adjoining root were individually separated from each other by tumor cells. The neoplastic cells were distributed along the sheath of Schwann in much the same way as they were in the leptomeninges of the cervical cord and above. The arrangement of a few tumor cells around a nerve fiber produced scattered, suggestively whorled structures within the growth. No glial fibers were found in sections of the tumor stained by phosphotungstic acid hematoxylin.

There was a moderate amount of collagen and reticulum throughout virtually all parts of the tumor, which was considered to be almost entirely preexisting fibers from the sheath of Schwann, endoneurium, and perineurium of nerves permeated by tumor. Where the tumor tissue was not invading nerves, there was only a minimal amount of reticulum around a few blood vessels.

Case 2. F–1062: This animal was a brown, female mouse of the NHO strain subline BrS, age 12 months at the time of autopsy. It had been exposed to a total of 1,000 r whole-body radiation given in 12 consecutive daily treatments, before the age of 2 months. On the day of autopsy the hind legs were paralyzed and there was difficulty in breathing. The presence of a tumor of the central nervous system was suspected, and the

animal was killed by ether. The physical factors were: 140 KVP, 3 mm. AL added filtration, 30 cm. distance, 42 r per minute.

Combined anatomic and histologic diagnoses: primary tumor in spinal canal involving meninges, cord, spinal roots, and ganglion, a lumbar spinal nerve, with infiltration of nerve bundles emerging from canal and surrounding muscle; papillary adenomatous cyst and tubular adenoma of ovary; Klossiella muris infestation of kidney and pyelonephritis; dilatation of urinary bladder, marked; brown degeneration of adrenal gland and cortical adenoma, small; cystic hyperplasia of uterine endometrium and focal acanthosis of cervix; hemosiderosis of spleen; dilatation of gastric glands; interstitial pneumonia; and focal calcification of tongue.

Following removal of the viscera, the carcass, with spinal cord intact, was fixed in acid Zenker's fluid. The cord was not removed but instead multiple blocks of tissue were taken from the vertebral column at different levels in order to include a tumor if present. These sections also contained spinal cord and the muscles, nerves, and other tissues of the posterior abdominal wall and back. The same histologic methods were employed as in Case 1.

A section through an upper lumbar vertebra revealed the largest cross-sectional area of tumor (fig. 13). This occupied approximately one half of the spinal canal. Much of this neoplastic mass was within the dura, occupying the lateral half of the canal. In areas it infiltrated the epidural tissues and in two places extended into the cancellous bone of the dorsolateral wall of the spinal canal. There was neoplastic infiltration of several spinal roots and one spinal nerve, a spinal ganglion, and a narrow border of the lateral half of the cord, as well as the leptomeninges and dura. A thin sheet of neoplastic cells, infiltrating the meninges, crossed the midline at this level, dipping into the anterior fissure and Virchow-Robin spaces (fig. 15). The spinal ganglion, spinal nerve, and meninges on this side were intact and not infiltrated by tumor.

A section through the level of the upper limits of the cauda equina (fig. 14) showed minimal neoplastic permeation of several nerve fibers, of the leptomeninges of the lateral and ventral portion of the cord, and of the anterior fissure. A spinal nerve, and a spinal ganglion within the intervertebral foramen and the spinal nerve, and many peripheral nerves in the surrounding musculature were extensively invaded by tumor (figs. 14 and 16). As in Case 1, the neoplastic infiltrate in the peripheral nerves was usually self contained but in places extensively invaded muscle.

Sections through the lower end of the cauda equina showed no evidence of tumor.

The tumor cells where they infiltrated nerves were sometimes arranged in whorl-like formation with a centrally placed nerve fiber (figs. 17 and 18). In the areolar tissue about the nerves the tumor cells were loosely arranged and tended to be large as compared with the infiltrate in muscle, where they were often spindle-shaped and occasionally palisaded. Most of the tumor cells were uniform in size, and possessed a single nucleus and an abundance of rather basophilic homogeneous cytoplasm. Many cells

were elongated; others were round, angular, or spindle shaped. The nucleus was round, oval, elongated, or horseshoe-shaped, and occasionally excessively lobated or folded. It was usually moderately hyperchromatic and less frequently pale and vesicular, with the chromatin material arranged as strands and fine granules and rarely as a single large clump. Occasionally there were two nuclei or a single, large, lobated nucleus. Mitotic figures averaged one per four or five oil-immersion fields. Except for one moderately large area of necrosis and hemorrhage, with infiltration of phagocytes, regressive changes were minimal and scattered. The neoplastic cells tended to be distributed intimately along the sheath of Schwann in the affected nerve bundles and, in the spinal ganglion, widely separated the ganglion cells, many of which were compressed, degenerated, and necrotic.

Collagen and reticulum were largely absent from the tumor where the neoplastic cells were proliferating and disengaged from other tissues, but were abundant where nerves were invaded, representing in these latter locations preformed fibers. The phosphotungstic-acid-hematoxylin stain did not reveal glial fibers in the tumor.

DISCUSSION

The classification of these tumors with respect to cell of origin presented difficulties. Both tumors arising in mice of the same inbred strain were so similar in their location, biologic behavior, and histopathologic pattern that they were in all probability identical in origin. From the appearance of the main mass of the tumor in each case, the site of origin appeared to be within the spinal canal and in intimate association with nerve roots and meninges. In each case the main mass of the tumor occupied a chiefly ventrolateral position within the spinal canal. The possible cells of origin within the canal were therefore from the leptomeninges, glia of spinal cord, capsule cells of dorsal root ganglion, Schwann cells and endoneurial fibroblasts of dorsal and ventral roots and spinal nerve, vascular endothelium, and reticuloendothelium. Although in each case the spinal cord showed permeation by neoplasm, this was limited to a few Virchow-Robin spaces and the meningeal surface which was in relation to the principal mass of tumor, and appeared to be secondary. Moreover, the phosphotungstic-acid-hematoxylin stain showed no glial fibers. These considerations would serve to rule out the glia as the source of the tumor. The likelihood of the tumor being a sympathicoblastoma or neuroblastoma from the spinal ganglia was eliminated on the basis of histologic pattern. The possibility of hemangioendothelioma and reticulum-cell sarcoma was considered unlikely on the basis of histologic pattern. Endoneurial fibroblast was eliminated as a source by absence of fibroglia and new-formed reticulum. This narrows the possibilities to the meninges proper or the sheath of Schwann, which is anatomically continuous with the arachnoid. In its manner of spreading, the tumor tissue exhibited a pronounced affinity for meninges and nerves. Both tumors grew in a sheetlike fashion along the meninges, extending into the depth of the anterior fissure of the cord and

into the Virchow-Robin spaces. From its presumed origin within the spinal canal the neoplasm in each case extended through the intervertebral foramina, invading the spinal ganglia and corresponding peripheral nerves for a considerable distance. It infiltrated the skeletal muscle and fat surrounding these involved nerves to only a limited degree in Case 1, and to a more marked degree in Case 2. This affinity for nervous structures supports the belief that the tumor originated from nervous tissue, very possibly dorsal spinal roots. As for histologic pattern the only characteristic feature was the formation of suggestive whorls, but these were considered to be due to the distribution of tumor cells around an individual nerve fiber and not true whorls characteristic of a meningioma. In addition, in a few sites the tumor cells showed palisading. In summary then, the evidence was compatible with an origin from the meninges or the sheath of Schwann. Between the two there is little choice, although admittedly it would be unusual for a primary meningeal tumor to invade nerve roots and peripheral nerves.

Neoplastic compression of the spinal cord in mice has been occasionally encountered at autopsy due to (*a*) leukemic infiltration extending from the vertebral marrow, (*b*) secondary invasion by spontaneous or induced tumors primary in the skin and subcutaneous tissues of the back or of adjacent viscera, or (*c*) transplanted tumors in this region. Neither of the mice in this study had a malignant primary tumor which might have yielded secondary deposits in the spinal canal. If the tumor arose from the meninges, it developed primarily within the spinal canal and secondarily invaded the peripheral nerves. If on the other hand the tumor arose from the sheath of Schwann, the primary growth might have developed either within or without the canal or possibly in both locations simultaneously. The only evidence that can be brought to bear on these latter possibilities is that the anatomical appearance of the lesions in each mouse strongly supported the belief that the primary growth arose within the spinal canal, with secondary invasion along the spinal nerves emerging from the cord at or near the level of the main mass of the tumor. This negative evidence for a primary site other than the tissues of the spinal canal, together with the more positive evidence from histopathologic studies, further supports the belief that the neoplasm arose within the canal from the meninges or the sheath of Schwann.

Clinically, a neoplasm compressing the spinal cord was suspected in each case from the signs of peripheral-nerve paralysis. It is therefore evident that if this type of tumor occurred with any degree of frequency the chances are it would not be overlooked by investigators who examine their experimental and control mice clinically. In our own laboratory we have not observed mice showing evidence of cord compression and spinal-nerve involvement except from secondary neoplastic deposits as mentioned. These are the first primary tumors apparently arising within the spinal canal that have been found in the material examined at the National Cancer Institute since its establishment.

The finding of exceedingly rare identical tumors in two mice of the same inbred strain raised the question whether this lesion would not be found in other animals of this strain if they were carefully examined with this point in mind. The NHO strain of mouse was originated by Dr. L. C. Strong. The present observations were based upon a study of a small group of NHO mice which had been released to Dr. Henry S. Kaplan for other studies (*3*). In the future, it is suggested that any investigator having mice of this strain observe them for the possible development of such tumors.

One of the two mice in this report was untreated. Hence, the tumor in this case was spontaneous. The other mouse received whole-body radiation. In view of the relative insensitivity of the tissues of the nervous system to the effects of roentgen radiation it is probable that the experimental procedure of whole-body radiation played no role in the development of the tumor in the second mouse.

Another point that deserves comment is the clinical and pathologic correlation. In the first animal there was paralysis affecting the left foreleg and left hind leg. The tumor occupied the left anterolateral aspect of the cervical spinal canal and extensively infiltrated the spinal nerves emerging from the intervertebral foramina on the left and forming the brachial plexus. In the second case both hind legs appeared paralyzed. As compared with Case 1, in this second case the mass of tumor within the spinal canal was more caudal, being located in the lumbar region, and was much larger, resulting in obviously greater compression of the cord. In both cases there was dilatation of the bladder compatible with a cord bladder. So striking were the clinical and pathologic manifestations that it would be difficult to overlook such spinal cord tumors, if they developed with any degree of frequency in a given strain of mice which were under careful clinical scrutiny.

SUMMARY

A report is made of two mice of the NHO strain with identical nervous tissue tumors probably arising from the meninges or from the sheath of Schwann within the spinal canal.

The presence of a tumor in this location was suspected in both mice clinically.

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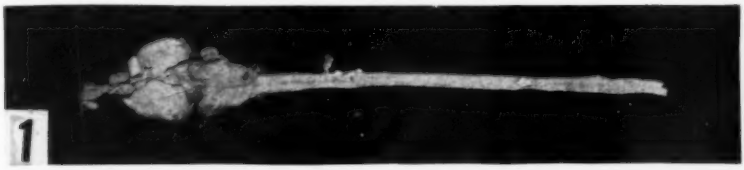
PLATE 15

FIGURE 1.—Ventral surface of brain and spinal cord showing tumor nodule in cervical region. Photograph of gross specimen.

FIGURE 2.—Ventral surface of spinal cord showing tumor in cervical region. Photograph of gross specimen. Slightly enlarged.

FIGURE 3.—Section of spinal cord and tumor. Photomicrograph × 30.

FIGURE 4.—Spinal cord showing tumor infiltrating ventral sulcus and Virchow-Robin space. Photomicrograph × 210.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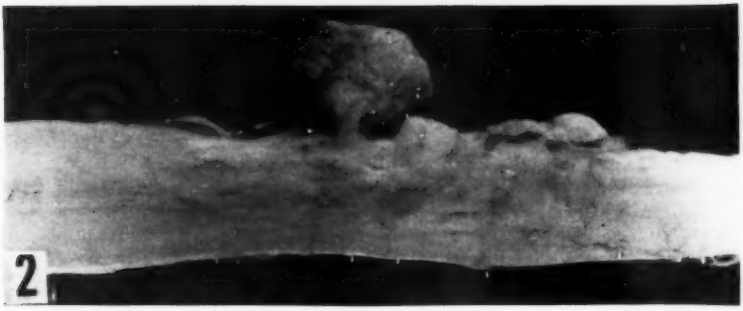
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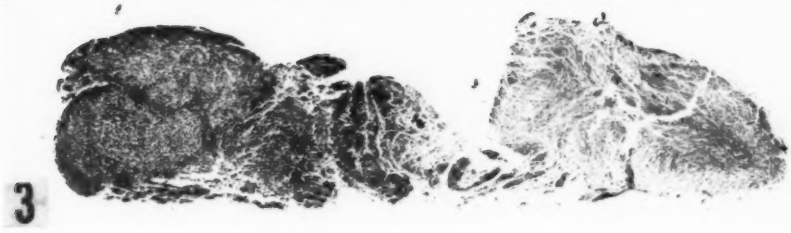
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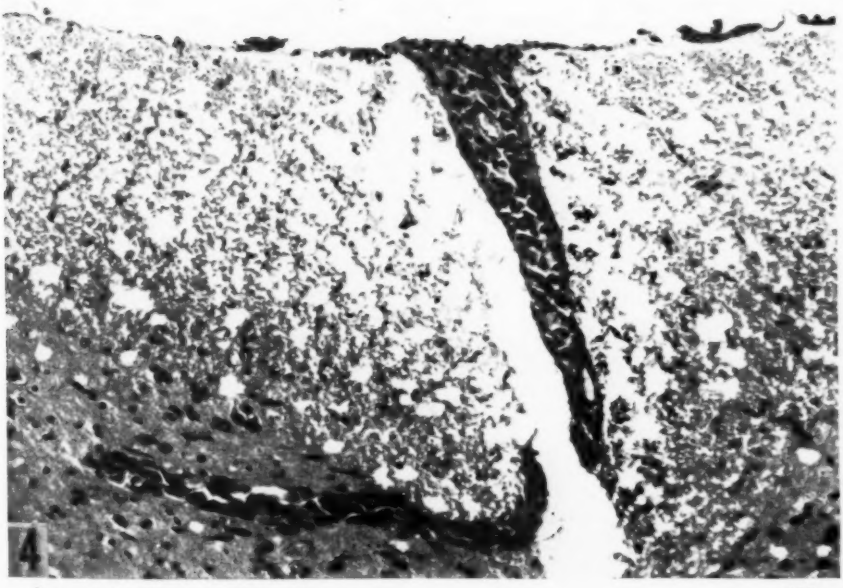
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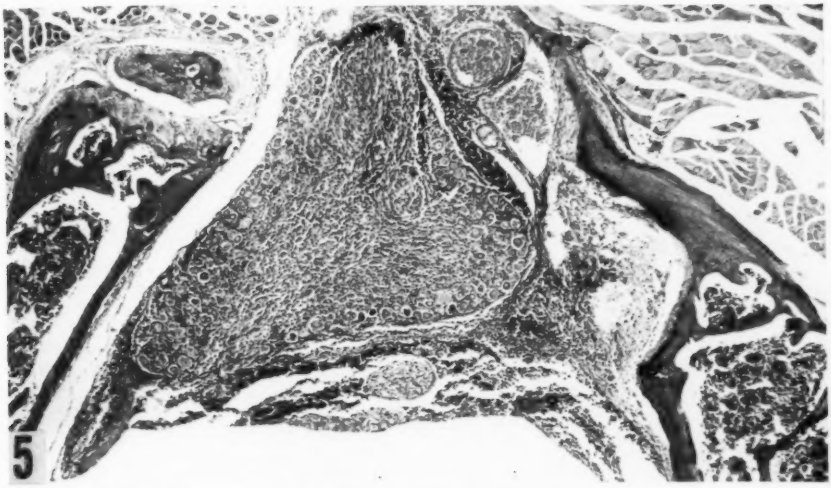
PLATE 16

FIGURE 5.—Section through bony wall of the left anterolateral portion of the spinal canal after removal of cord. A spinal nerve, spinal ganglion, and the meninges are invaded by tumor. Photomicrograph × 58.

FIGURE 6.—Peripheral nerves of left cervical plexus ventral to scapula extensively invaded by tumor. Medially (right border of illustration) there is an area of hemorrhage in soft tissues. Photomicrograph × 25.

FIGURE 7.—Section of nerve shown in figure 6 (area outlined in square), showing permeation by tumor. Photomicrograph × 210.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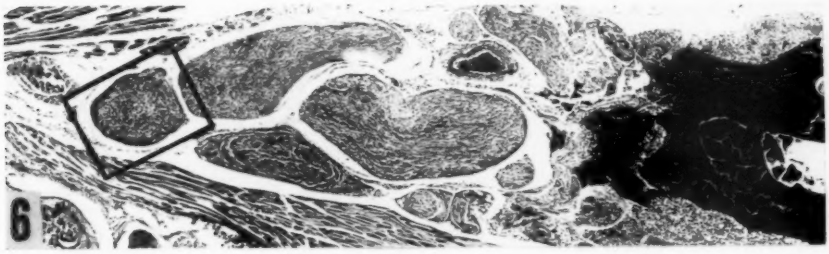
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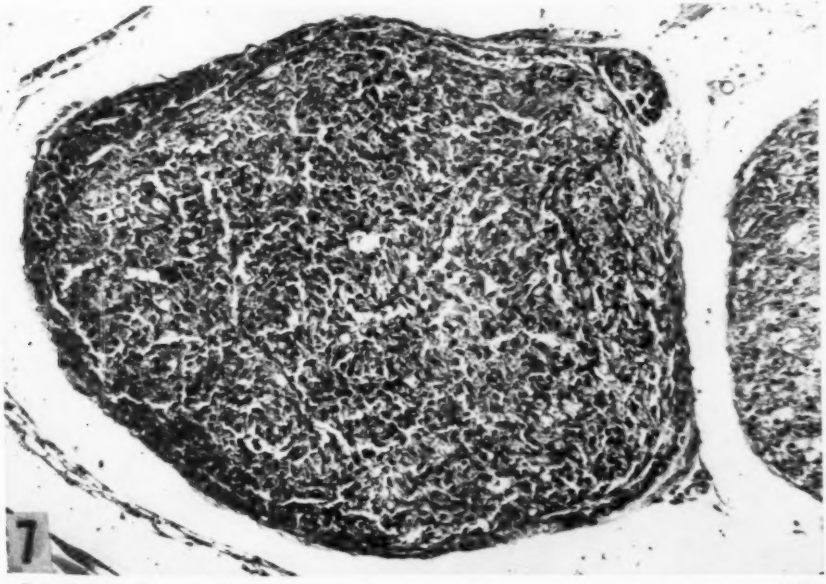
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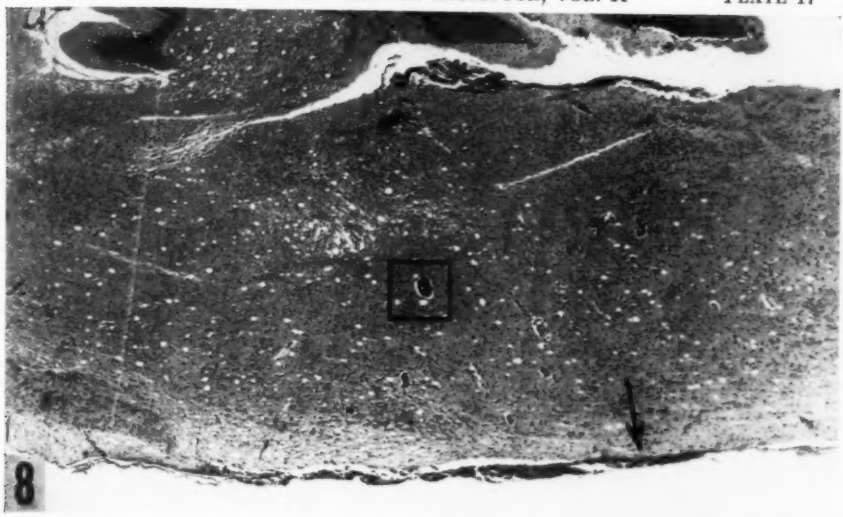
PLATE 17

FIGURE 8.—Brain stem showing meningeal spread of tumor, ventral surface (arrow), and several foci of tumor in spaces of Virchow-Robin (see square). Photomicrograph × 35.

FIGURE 9.—Higher power of above illustration (arrow), showing neoplastic infiltration of meninges over ventral surface of brain stem. Photomicrograph × 650.

FIGURE 10.—Infiltration of neoplasm in Virchow-Robin space from area within square, figure 8. Note small compressed vessel in center of tumor deposit. Photomicrograph × 610.



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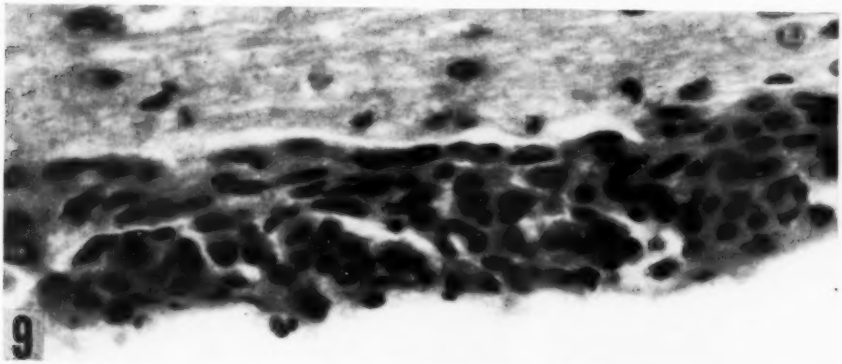

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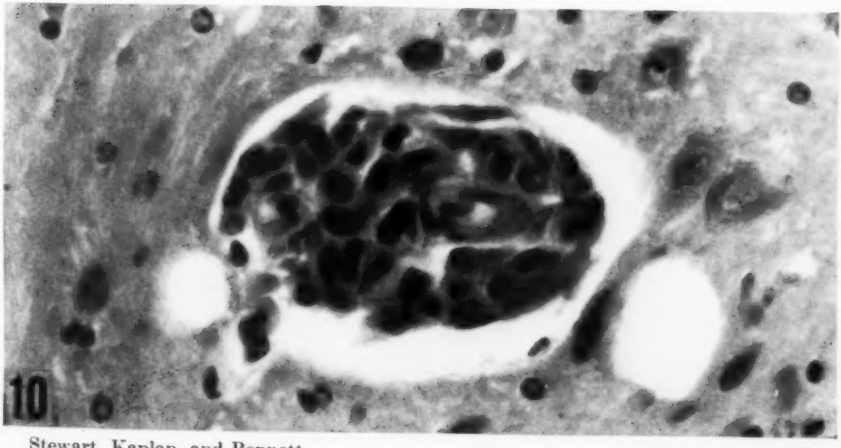

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PLATE 18

FIGURE 11.—Immediately above and below the center of the field are transverse sections of two nerve fibers (pale areas), the peripheral limits of which are outlined by the whorl-like arrangement of the tumor cells. Photomicrograph × 1,680.

FIGURE 12.—Area of the tumor showing appearance of the neoplastic cells. Photomicrograph × 900.

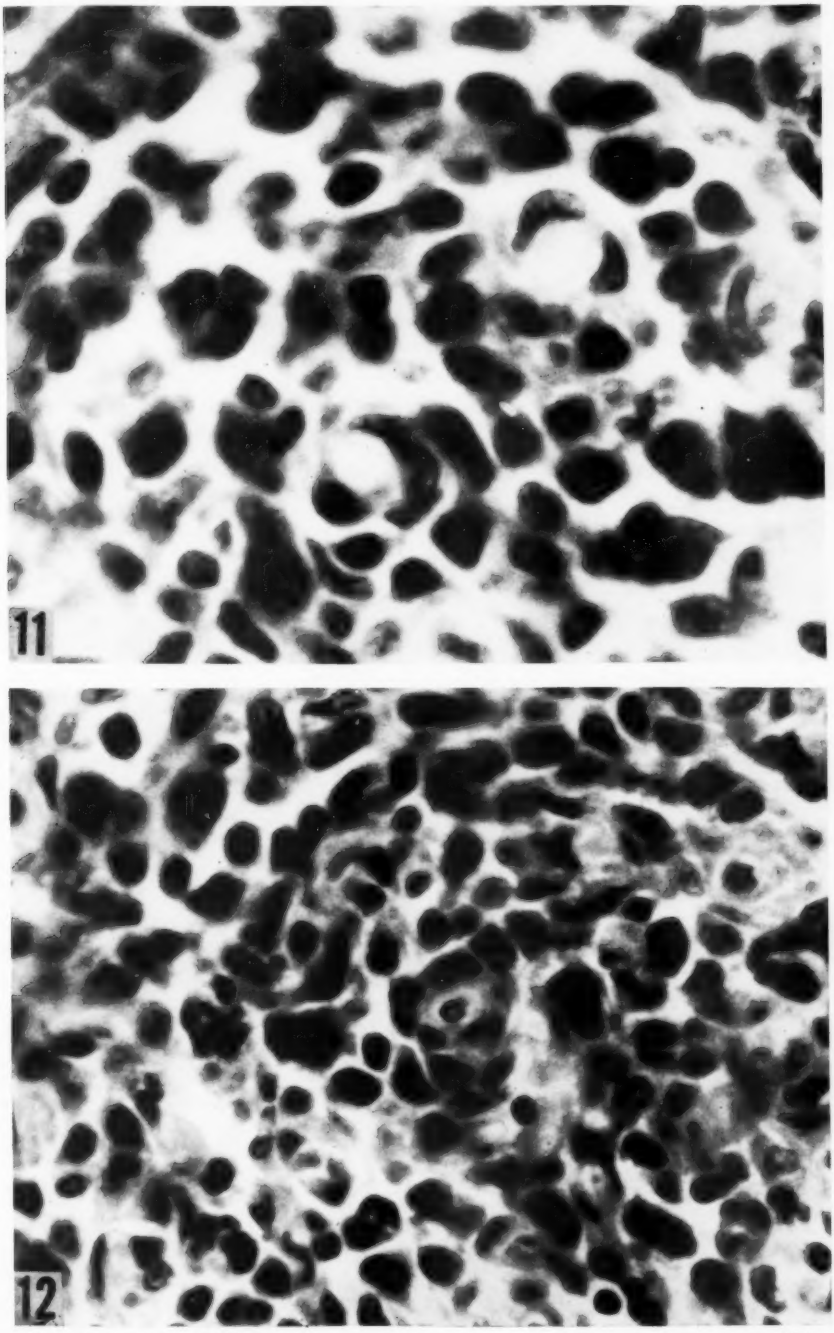

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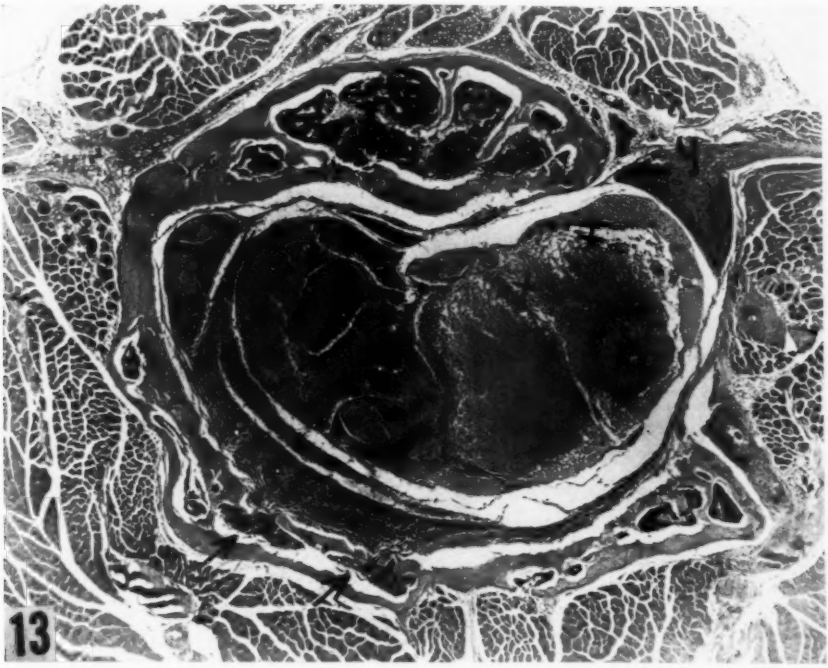
PLATE 19

FIGURE 13.—Transverse section of upper lumbar vertebra. Approximately one half of the spinal canal is occupied by tumor, in which many nerve bundles are recognizable. In the lower left portion of the illustration the tumor penetrates the marrow space in two places (arrows). The cord is compressed and infiltrated to a limited extent on the side nearest the tumor mass. The tumor infiltrates the anterior fissure (x) and extends across the midline by way of the meninges (x). The spinal nerve and ganglion (y) on this side are not involved by tumor. Photomicrograph × 35.

FIGURE 14.—Transverse section of bony wall of vertebral canal at beginning of cauda equina, a lower level than shown in the above figure. Occupying the foramen are a spinal nerve and spinal ganglion extensively infiltrated with tumor. In the adjacent muscle are several cross sections of nerve bundles infiltrated by tumor which also permeates the surrounding muscle. Within the canal the nerves and meninges are infiltrated by tumor tissue. Photomicrograph × 45.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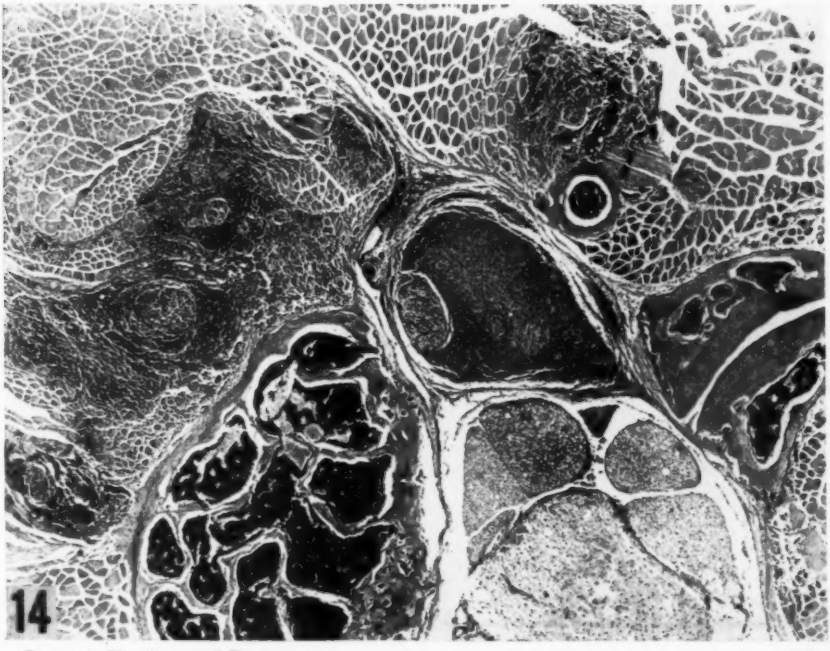
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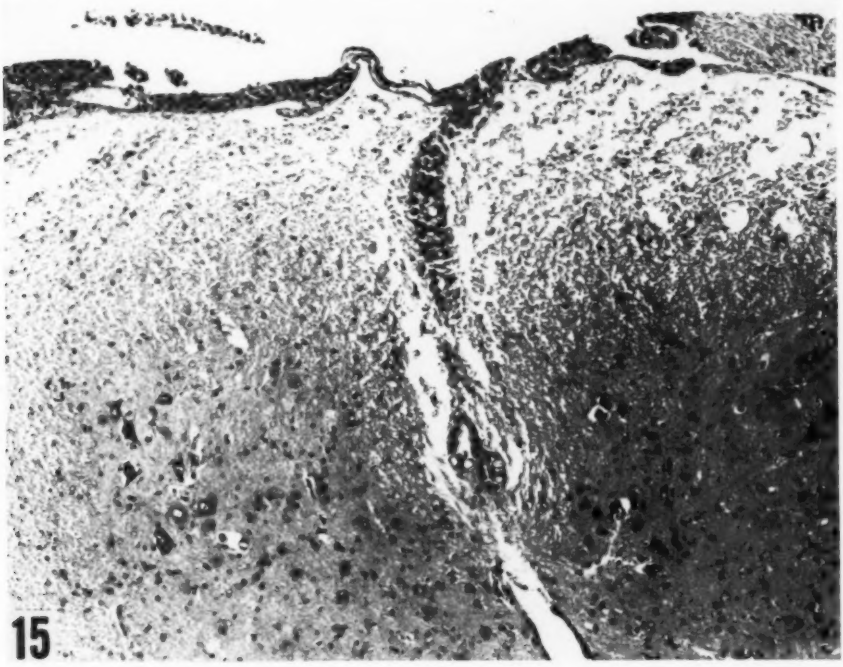
PLATE 20

FIGURE 15.—Spinal cord showing tumor tissue infiltrating the meninges and spreading into the ventral sulcus. Photomicrograph × 110.

FIGURE 16.—Section of peripheral nerves showing tumor which extends into surrounding muscle. Photomicrograph × 210.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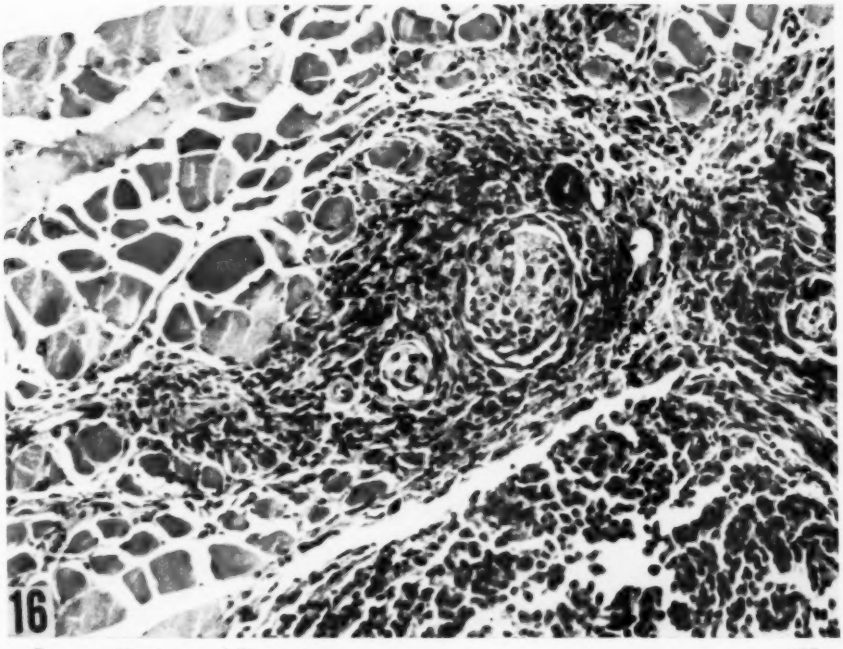
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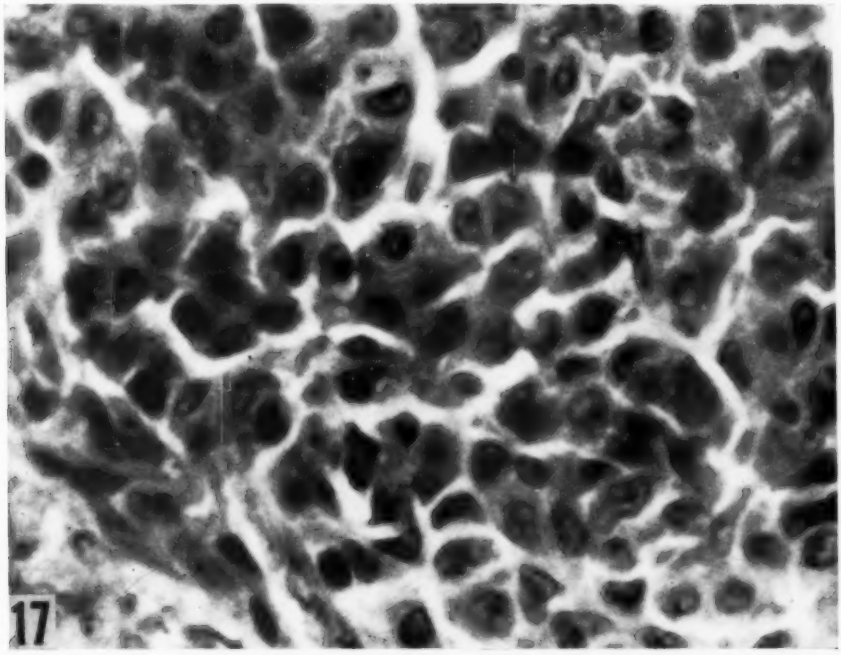
PLATE 21

FIGURE 17.—Area of tumor showing appearance of neoplastic cells. Photomicrograph × 1,000.

FIGURE 18.—A little to the right of and above and below the center of the field are cross sections of two single nerve fibers showing the whorl-like arrangement of the tumor cells about them. Photomicrograph × 1,600.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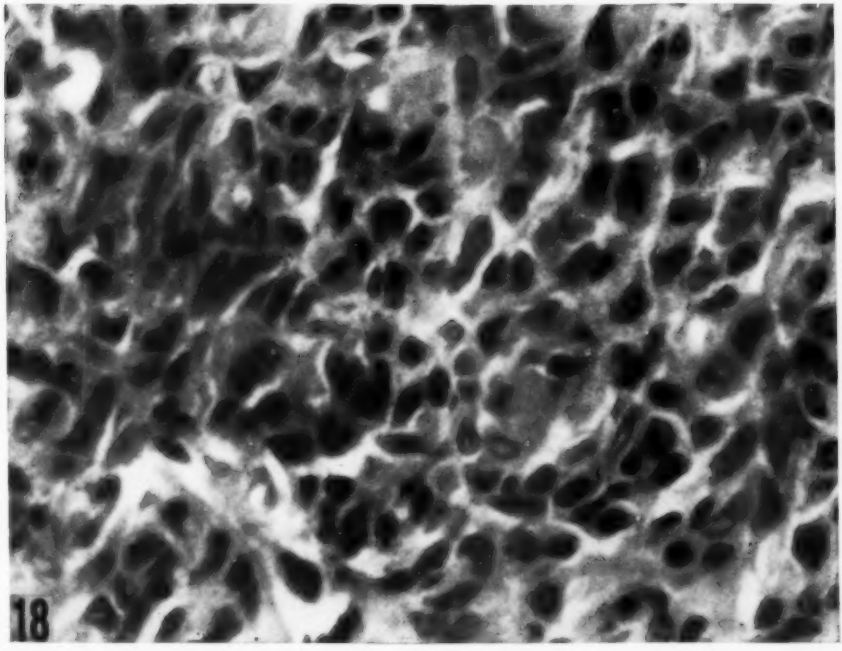
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CHROMATOGRAPHIC STUDIES ON THE SEPARATION OF THE VIRUS FROM CHICKEN TUMOR I. I. EFFECT OF SALT CONCENTRATION ON ADSORPTION, ELUTION, AND PURIFICATION [1] [2]

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The highly selective capabilities of chromatography have been demonstrated in many ways, including simultaneous separation of 22 amino acids (*1*), the reported separation of isotopes (*2*), stereoisomers (*3*), the homologous straight chain fatty acids (*4*), and antibiotics, such as penicillins (*5*) and streptomycin (*6*).

Confronted with a difficult purification problem of separating a virus-like agent from morphologically similar normal constituents, the question arose as to whether or not the principles of chromatography could be applied to the separation of particles in the macromolecular category with "molecular weights" of the order of 140 millions. Previous applications had been limited to soluble molecules of ordinary size, so that it was unknown whether the forces involved in chromatography would operate at this higher level. Since the chicken tumor agent is pathologically "tagged," it is subject to positive identification and quantitative measurement and, though colorless, can thus be followed on a chromatographic column. This paper, together with others in the series, supplements the brief report published earlier (*7*) and describes with some detail the chromatographic behavior of an intracellular particle, the agent of chicken tumor I.

Since there are several comprehensive texts and review articles available on chromatography (*8–16*), only a brief discussion of the principles employed in these experiments as compared with flask adsorption, sometimes used for virus purification (*17–23*), is undertaken.

The advantage of the chromatographic column over the conventional procedure of flask adsorption is somewhat analogous to the advantage of fractional distillation over simple distillation. In a flask, adsorption of the various components takes place to the degree described by their partition or adsorption coefficients. Depending upon several factors, the unit adsorbent particle will have various amounts of the different components attached to it. Separation of adsorbent and solvent at this stage gives a bifurcation of the mixture but not necessarily a resolution of any one component. While a single substance may be isolated if its

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[2] Presented in part at the meetings of the American Association for Cancer Research, Chicago, May 16–17, 1947.

[3] Statistical advice and services were provided by Mr. Jerome Cornfield, and statistical computations in the analysis of variance of the bioassays were performed by Miss Virginia Voorhees.

adsorption affinity is considerably different from the other components, the rest of the materials will remain unresolved.

However, in chromatography, the components may be adsorbed and desorbed repeatedly during the percolation down the column, and consequently substances with but slight differences in their partition or adsorption coefficients, under proper conditions, become spatially separated in the course of the operation. This procedure has been described as a countercurrent application of batch adsorption and is thus analogous to fractional distillation with reflux, the Craig liquid-liquid extraction procedure, and other countercurrent separation processes (*24*).

One of the basic problems in applying chromatography to labile particulates lies in finding a suitable adsorbent and solvent system in which the component to be isolated can be adsorbed, developed, and eluted, without denaturation and loss of biological activity. The following experiments indicate the results of applying a mild chromatographic system of diatomaceous silica as an adsorbent, and aqueous sodium chloride of various molar concentrations as solvents, to the separation of the causative agent of chicken tumor I.

MATERIALS AND METHODS

Columns, Adsorbents, and Solvents

In the experiments reported here small micro-columns, 8 mm. inside diameter and approximately 280 mm. in length, were employed. These were adapted from 10-ml. pipettes by cutting off the tips and flaring slightly to form a funnel which simplified the addition of adsorbent and solvents. In use, the tube was clamped in a vertical position and a plug of cotton introduced through the upper funneled end and forced with a rod to the constriction at the bottom to form a base for the adsorbent. In some experiments a few milliliters of filter-paper suspension were added to form a filter-paper cap over the cotton base.

The adsorbent in all experiments was silica in the form of diatomaceous earth, and sold under the trade name of Celite.[4] Two grades were used, Nos. 501 and 503, both of which have flow rates suitable for the colloidal solutions employed. The flow rate for adsorption, development, and elution of the agent, employing the small column, varied from 0.3 to 3 ml. per minute.

In all experiments reported, the adsorbent was suspended in 0.9 percent saline or distilled water and introduced into the column as a slurry. Further details are given in the description of the specific experiment. The house vacuum, of about 25 inches of mercury, was used to sediment the Celite in the columns and to aid the percolation of the tumor extract and solvents through the adsorbent.

Solvents[5] employed in these experiments were limited to aqueous sodium chloride of various molar concentrations, and triple distilled water.

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[4] Johns Manville Corporation, Philadelphia, Pa.

[5] Strictly speaking, the agent in question may be considered in suspension or in colloidal solution.

Tumor Agent

The virus-like agent (Rous virus) of chicken tumor I (Rous sarcoma) (*25*), was used on the columns as a partially purified suspension prepared by a procedure previously described (*26*). The source material for all experiments was thawed tumor tissue that had been grown by cell passage in the wings of 6-to-10-week-old chickens under carefully controlled conditions. The tumors were harvested aseptically after 7 days of rapid growth and stored at −40° to −60° C. for several weeks until used for an experiment.

Since the lability of the agent is presumably less at reduced temperature, all experimental operations were carried out within a range of from 2 to 15 degrees Centigrade.

Bioassay of Tumor Agent

Bioassays were carried out employing the balanced experimental design of Bliss and Marks previously described (*27*, *28*). For each assay of tumor-agent activity 2 dilutions of tenfold separation of the "unknown" were compared with 2 similar dilutions of a standard reference material in a balanced determination involving 4 inoculation sites on each of 40 chickens. Each dilution was tested an equal number of times at each inoculation location; there were, therefore, 40 observations on each dilution, 80 on each material, and 160 for each comparison of unknown and standard, or bioassay determination. The biological response used in making the tests was the reciprocal of the latent period [6] times 100 (100/LP), which has been previously described (*29*, *30*).

The concentration of tumor agent in a given fraction is expressed as percent potency [7] compared with a standard, which in most experiments was the partially purified starting material. In the semiquantitative experiments, relative potency is indicated by the latent period reciprocal means times 100, with no attempt to estimate the standard error involved. The statistical errors of all quantitative experiments were estimated by use of the analysis of variance.

For purposes of animal inoculations, the diluent for the virus fractions in all experiments was serum-buffer solution prepared by adding normal rabbit serum to 0.005 molar phosphate buffer, pH 7.0, to give a 2 percent serum concentration. The volume of the dose for all sites was a constant, arbitrarily set at 0.2 ml.

Chickens

All chickens used as test animals were from a single breed (New Hampshire Red, Christie strain) and were used for assay between 3 and 6 weeks of age. For a given experiment the chicks were from a single hatch, with one exception in which two groups 4 and 6 weeks of age were pooled. A

[6] The "latent period" is the interval between the injection of the virus fraction and the palpable or visible appearance of a progressively growing tumor. It is thus the incubation period and is not intended to imply virus latency in the usual sense.

[7] The use of the term "potency" in this paper refers to the concentration or quantity of viable agent in one preparation relative to that in another and is not intended to imply alterations in the virulence or infectivity of the virus. Although, if enhancement occurred through removal of an inhibitor or other unknown operation, it would be reflected in these experiments as an increase in concentration.

previous report (*31*) has shown that there is no significant difference in response to the agent between chicken groups of this age range.

Nitrogen and pH Determinations

Total nitrogen determinations were made by the micro-Kjeldahl method of Koch and McMeekin (*32*), employing direct Nesslerization. The relative color intensity between standard and unknown was determined by an abridged spectrophotometer (Aminco, type F) using a number 46 filter with an effective wave length of 458 mμ.

Appropriate volume adjustments were made so that the total nitrogen in most determinations was between 0.01 and 0.1 mg., with the color intensity falling between 40 and 90 percent light transmission.

Hydrogen-ion determinations were made with a Beckman or Macbeth glass and calomel electrode, pH meter.

EXPERIMENTAL PROCEDURES AND RESULTS

Brief mention of the use of the chromatographic column for virus purification was included in a previous report (*31*). In those studies flowing chromatography was employed, using a phosphate buffer system that gave a preparation containing more active agent per unit of nitrogen than any other method of purification used by us at that time. The results seem to justify a systematic effort to determine the controllable factors involved in the separation. For simplification, the following studies were undertaken with a single salt, sodium chloride, rather than the phosphate buffer complex.

(*a*) *Effect of Celite on the latent period of tumors.*—Under some conditions of elution, small quantities of fine Celite are released with the agent. The possibility of artificial enhancement of tumor production by this foreign substance was considered. The results of two semiquantitative experiments designed to determine the presence of any gross effect follow:

(1) Partially purified tumor agent was prepared and divided into 2 identical portions. Fine Celite was obtained by the elution of a blank column supported by a loose plug of absorbent cotton. The Celite suspension was added to 1 portion of the sample and an equal quantity of distilled water to the other. Equal log dilutions of −1 were prepared as usual in serum-buffer and the 2 fractions were injected into 20 chickens with a total of 40 sites.

(2) The second experiment was similar to the above with the exception that a column eluate was included in which precautions had been taken to eliminate Celite contamination by the use of a filter-paper cap over the cotton plug. The cap was deposited with negative pressure from a filter-paper suspension prior to addition of the adsorbent. Final log dilutions in this experiment were −2 to test the effect at lower agent concentrations. The results are shown in table 1.

In two out of three of the above comparisons the small differences in response favored the control fractions rather than those with added Celite, while the fractions in the other comparison were nearly identical. The

TABLE 1.—*Chicken response to tumor agent fractions with, and without, added Celite*

Experiment	Agent fractions	Number of sites	Log dilution	Mean tumor-agent activity (Reciprocal days x 100)
(1)	Agent	20	−1	18. 1
(1)	Agent plus Celite	20	−1	18. 3
(2)	Agent	18	−2	13. 6
(2)	Agent plus Celite	18	−2	12. 7
(2)	Agent eluate	18	−2	13. 8
(2)	Agent eluate plus Celite	18	−2	13. 1
(1) and (2)	Agent control fractions	56	−1 and −2	15. 2
(1) and (2)	Agent plus Celite fractions.	56	−1 and −2	14. 7

variation in mean response is well within the limits of error of the assay, and indicates that the magnitude of the effect of Celite contamination in the bioassay, where latent period is the criterion, is relatively small or absent under the conditions described in this paper.

(*b*) *General effect of salt molarity and pH.*—Exploratory experiments indicated that adsorption of the chicken tumor agent took place on silica columns in the presence of physiological saline and was apparently reversed by adding distilled water. To confirm these observations and to determine whether salt molarity or pH was responsible for this phenomenon two semiquantitative experiments were run. The procedures and their results were as follows: Partially purified tumor agent was concentrated at high speed in the centrifuge. The 6 identical pellets resulting were resuspended in a series of logarithmically decreasing molar concentrations of sodium chloride, ranging from 0.1 through 0.00001 molarity and terminating in distilled water. (A similar series of log dilutions was prepared in the second experiment starting from physiological concentration of 0.154 through 0.0000154 molar.) Six columns were prepared by suspending 2 gm. of No. 503 Celite in 25 ml. of each of the above solutions. Five milliliters (0.4 gm.) of adsorbent of each diatomaceous suspension were added to a microcolumn as described above. The adsorbent was deposited with vacuum, and the columns were washed with 10 ml. of the same solution used for suspension. The tumor agent was added to its matching column and passed through with the aid of a slight vacuum. Each column was then washed with 5 ml. of the appropriate matching solvent, which was collected with the agent filtrate. These percolates were tested at 128 sites in 32 chickens for approximate virus content.

Of 32 sites injected with the percolate from the 0.1 (and 0.154) molar columns, none produced tumors. (Dilution experiments indicate that less than one millionth of the virus from the starting material passed through these columns.)[8] The filtrate from the 0.01 (and 0.0154) molar

[8] In an unpublished experiment testing similar material the −6 log dilution gave 22 tumors out of 40 inoculation sites or 56.7 percent. The 50-percent-infectivity point, determined by bioassay for this agent, was −6.2 log dilution. On this basis, if one millionth of the starting agent had passed through the column, 16 to 18 of the 32 sites inoculated would have been expected to develop tumors.

TABLE 2.—*Effect of salt concentration on adsorption of tumor agent on Celite 503*

Experiment	NaCl molar concentration	Number of sites	Virus activity in percolates [1] (Mean reciprocal days x 100)	pH of eluent
(1)	0.154 (0.9 percent)	8	All negative	5.4
	0.0154	8	9.5 (weak)	5.5
	0.00154	8	13.5 (relatively potent)	5.7
	0.000154	8	12.8 (relatively potent)	6.0
	0.0000154	8	12.9 (relatively potent)	6.1
	Distilled water	8	14.3 (relatively potent)	5.9
(2)	0.154	12	All negtive	5.7
	0.1	12	All negative	5.7
	0.01	10	13.7 (weak)	5.6
	0.001	11	16.1 (relatively potent)	5.8
	0.0001	11	17.8 (relatively potent)	6.0
	0.00001	12	16.7 (relatively potent)	5.8
	Starting agent	12	17.0 (relatively potent)	---

[1] For purposes of orientation on the approximate relative amounts of virus eluted with the 0.01 molar as compared with 0.001 molar salt concentration, one may tentatively assume that the mean slope of the dosage-response curve as determined in quantitative bioassays would hold approximately for a semiquantitative determination. Thus, using the slope of 1.8, if the mean reciprocal of 13.5 is assigned the value of 100 percent virus eluted, the mean reciprocal of 9.5 would represent a virus concentration of the order of 0.6 percent. In experiment 2, 16.1 equaling 100 percent, 13.7 would be of the order of 5 percent.

The difference in potency level between the two experiments is due to a difference of agent concentration in the starting materials.

columns produced tumors, but the duration of the incubation or latent periods indicated that these materials were substantially weaker than the starting agent, and that adsorption for the agent was still functioning at this NaCl molar level. The high potency of the percolates from the other columns, 0.001 molar and lower saline concentrations, including distilled water, could not be differentiated from each other or from the potency of the starting-agent preparation in these experiments. The data are listed in table 2.

The effect of pH in a system of this type might be expected to be of critical importance, since it has been used to control adsorption and elution of the agent on alumina in flask-adsorption studies (*21*). However, the effects of fluctuation found in the weakly buffered saline solutions employed are listed in table 2 and indicate that within the restricted range shown the effect of adsorption and elution of the tumor agent is a primary function of salt concentration and is independent of minor pH differences. It is noted that complete adsorption was obtained in experiment (2) at 0.154 and 0.1 molarity with a pH of 5.7; whereas, in experiment (1), elution was obtained at the same pH but with the molarity reduced to 0.00154.

(*c*) *Adsorption and elution of agent and nitrogen without column development.*—The preceding data provided the basis for the following quantitative experiments. These differed from the above in that equal quantities of agent, in a common solvent, were adsorbed on identical columns and then each column was eluted with a different molar solution.

Partially purified agent (*26*) was added to five similar microcolumns, which were prepared by adding 5 ml. of a 10-percent slurry of No. 503

Celite in 0.154 molar (0.9 percent) sodium chloride solution. Each column was washed with 20 ml. of cold physiological saline to yield a column ready for agent adsorption 35 mm. high and 8 mm. in diameter, and containing approximately 0.5 gm. of adsorbent. Fifty milliliters of partially purified tumor agent in 0.9 percent saline were adsorbed on each column. The receiving flasks were changed, and 10 ml. of an eluting solution were put through the columns with the aid of negative pressure. Each eluent represented a different molar concentration of sodium chloride from a logarithmic series starting at 0.01 molar (−2 log molar),[9] decreasing to 0.00001 molar (−5), and terminating with distilled water. The columns were not developed between adsorption and elution in this experiment. This deficiency will be compared with succeeding experiments.

The 5 eluates represented the unknowns, and were titrated in 240 chickens against the partially purified starting material introduced on the columns. Nitrogen determinations were made on all fractions. The results of this and the 2 succeeding experiments are summarized in table 3.

This experiment demonstrated for the first time, on a reliable quantitative basis, that the tumor agent in the column eluates was concentrated and purified as compared with the partially purified starting material.

The data show a clear-cut difference between the eluates of −2 and −3 log molar salt concentration, both in quantity of agent released and its purity as expressed by the potency-nitrogen ratios. The potency differences have been tested for significance and could occur by chance less than once in 1,000 times (p is less than 0.001). The error terms for the potency values of the eluates from −3 log molar through distilled water overlapped, so that the experimental differences between these eluates could not be considered significant. Since the probable value of the potency-nitrogen ratio for the −3 eluate was the highest for the series, the possibility of an optimum in this vicinity was considered, and the two following quantitative experiments were performed.

(*d*) *Adsorption and elution of agent and nitrogen with column development.*—The procedure was the same as in the previous experiment, with the following exceptions: intermediate elution concentrations of −2.3 (0.005) and −3.3 (0.0005) molarities were inserted midway on each side of the apparent −3 log molar peak, and the −5 log molar and distilled water fractions were discontinued.

Development of the columns was introduced for the first time in this experiment. Following adsorption of the tumor agent, the columns were each washed with 10 ml. of 0.154 molar saline solution. Since adsorption of the agent on the column in the presence of this solvent had been demonstrated, this appeared to be a logical chromatographic procedure for removing unadsorbed, nonvirus material. The advantage gained is demonstrated in table 3 and in text-figures 1 and 2. The data show that there was a persistent decrease in percent nitrogen released from the columns in the elution range, whereas the potency was maintained at the level of

[9] For simplicity in handling, and because many of the characteristics found are convenient functions of log concentration rather than arithmetic concentration, the discussion is carried through in these terms.

TABLE 3.—*Effect of salt concentration on quantity of chicken tumor agent and total nitrogen eluted from Celite columns*

NaCl concentration of eluents			Nitrogen mg/ml.	Percent nitrogen	Percent potency [2]	Standard error of potency estimate [3]	Potency-nitrogen ratio [4]	Total agent recovery [5]
Molarity	Log molarity	Experiment [1]						
Starting material		(c)	0.0064	100.0	100.0		1.0	(100.0)
		(d)	.0074	100.0	100.0		1.0	(100.0)
		(e)	.0092	100.0	100.0		1.0	(100.0)
0.01	−2.0	(c)	.0012	18.7	19.2	+11.6, −7.2	.96	3.8
		(d)	.0012	16.2	2.1	+0.8, −0.6	.1	.4
		(e)	.0017	18.5	4.0	+1.7, −1.2	.2	.8
0.005	−2.3	(c)						
		(d)	.0016	21.6	18.5	+6.2, −4.6	.9	3.7
		(e)	.0013	14.1	20.4	+7.9, −5.7	1.4	4.1
0.001	−3.0	(c)	.0024	37.5	130.9	+49.2, −35.8	3.4	26.2
		(d)	.0026	35.1	66.3	+21.3, −16.1	1.9	13.3
		(e)	.0017	18.5	92.0	+28.7, −21.9	5.0	18.4
0.0005	−3.3	(c)						
		(d)	.0024	32.4	177.4	+61.0, −45.4	5.5	35.5
		(e)	.0025	27.2	138.3	+55.5, −39.6	5.1	27.7
0.0001	−4.0	(c)	.0041	64.1	104.9	+45.0, −29.9	1.6	21.0
		(d)	.0024	32.4	143.1	+46.9, −35.3	4.4	28.6
		(e)	.0033	35.9	145.7	+56.0, −40.4	4.1	29.1
0.00001	−5.0	(c)	.0057	89.1	203.3	+99.3, −66.7	2.2	40.7
		(d)						
		(e)	.0024	26.1	198.6	+75.7, −54.8	7.6	39.7
Distilled water		(c)	.0048	75.0	184.2	+84.8, −58.1	2.4	36.8
		(d)						
		(e)	.0046	50.0	210.4	+82.6, −59.3	4.2	42.1

[1] Experiment (c), columns not developed; experiments (d) and (e) both developed prior to elution.
[2] Percent potency is expressed as virus per unit-volume as compared with the partially purified starting extract. Values over 100 represent concentration.
[3] This should be interpreted as follows: The probability is approximately 2 to 1 that the true potency percent lies within the limits obtained by adding the plus figure (to the percent potency estimate) and subtracting the minus figure.
[4] Potency-nitrogen ratio equals percent virus divided by percent nitrogen to give an approximate index of purity based on nitrogen compared with the partially purified starting material.
[5] The eluent equaled ½ the adsorbed volume. Total recovery of starting agent is estimated by dividing the percent potency of the eluate by 5.

experiment (c). In text-figure 2, the experiments are compared for relative purification, and it is seen that there was a substantial and consistent gain in the potency-nitrogen ratios in the elution range.

Relatively strong adsorption for the agent was demonstrated at the −2 log molar level, and a moderated adsorption at the intermediate log molarity of −2.3; with the potency-nitrogen ratio being somewhat less than the starting agent. The apparent salt optimum in this experiment was at −3.3, with −4.0 log molarity following closely. Both of these values were higher than the −3 peak in the previous experiment, where no development of the columns was employed. Because of this technical difference between the two experiments, the question of an optimum eluent concentration was not interpretable.

At the log molarity of −3.3 and −4 NaCl the virus was concentrated to give a higher potency than the starting material and a purification increase of 5.5 and 4.4 times. Total virus recovery was 35.5 and 28.6 percent in these fractions.

(*e*) *Concentration, purification, and recovery over a wider saline range.*—This quantitative experiment was performed in essentially the same way as the preceding one except that the salt molarity range was extended to include −5 log molarity and distilled water. The results are summarized in table 3 and compared with the other experiments in text-figures 1 and 2.

Again the virus was concentrated in the −3.3, −4.0, −5.0 log M, and distilled water fractions, and each showed a purification increase ranging from 4.1 to 7.6 times the partially purified starting extract. Total virus recovery was an increasing function from 18.4 to 42.1 percent.

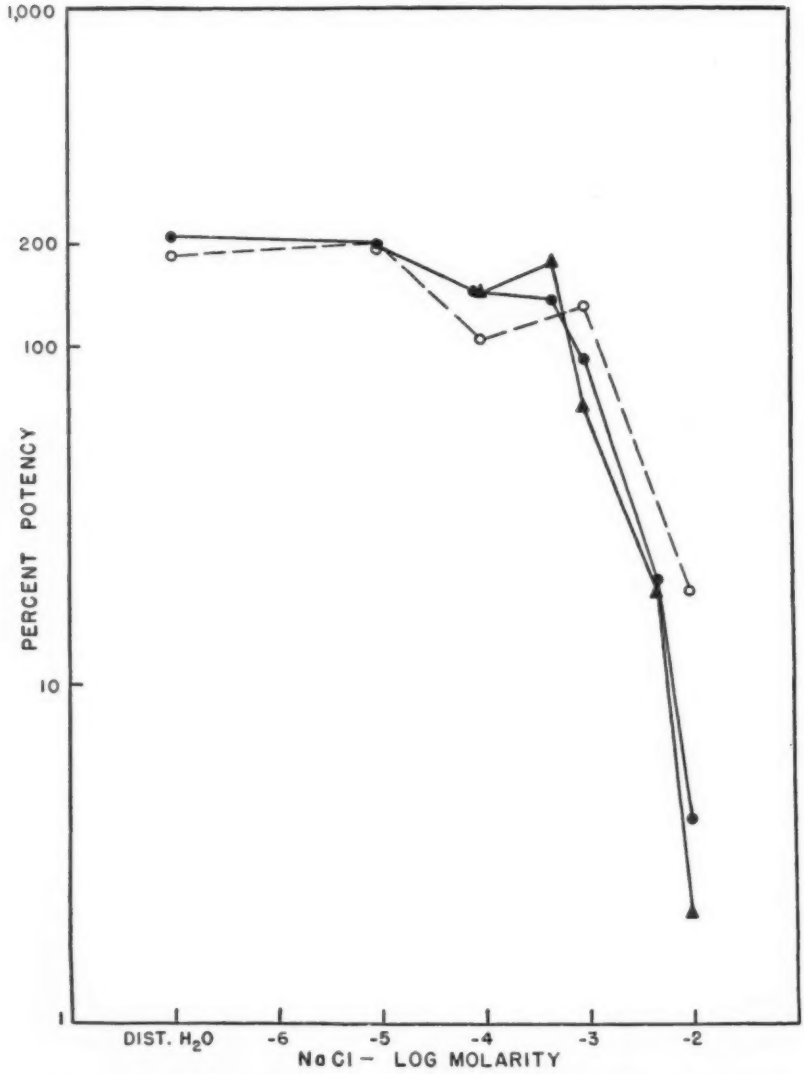
Text-figure 1 illustrates the relative percent chicken tumor agent eluted by the various molarities in the three quantitative experiments. Log potencies are plotted against log molarities. It may be noted that the potency level of the eluates from the last two experiments, in which the columns were developed, is maintained in spite of the substantial nitrogen reduction as compared with the undeveloped experiment. Thus, as might be predicted for an effective chromatographic system, development of the column removes some of the faster moving nitrogen components without interfering with the subsequent removal of the agent.

Text-figure 2 shows the potency-nitrogen ratios for the three experiments. The ratios are plotted on a log scale as in text-figure 1. Examination of the graph shows that in all experiments there is a sharp increase in the relative purity of the eluates from the −2 to the −3 log molar concentration. In the vicinity of −3 or −4 log molarity the ratio increase tends to level off, with the potency-nitrogen ratios of all lower concentrations appearing to fall either on a plateau or the top part of a parabola-like curve.[10]

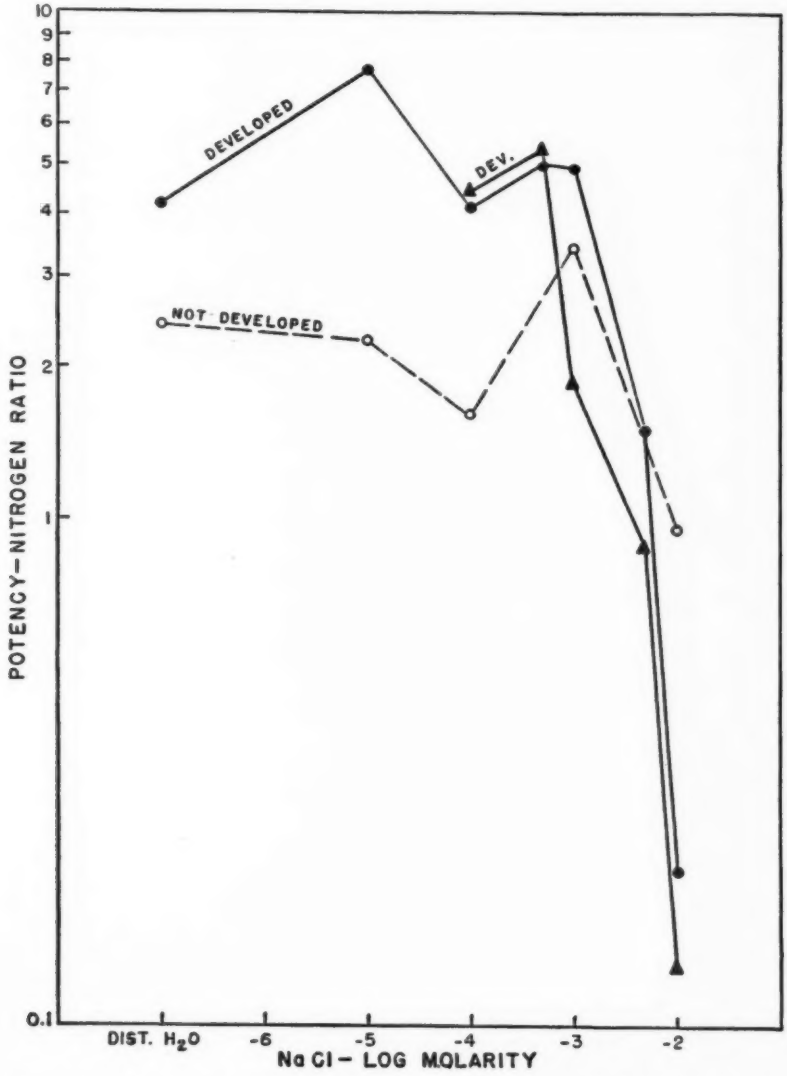
Text-figure 3 shows the relative purity obtained, based on nitrogen and turbidity, with the different salt concentrations. The striking increase from −2 to −3 log molarity is highly significant statistically, as are the potency-nitrogen points from −3 through distilled water when compared with the partially purified starting extract at unity. The experimental differences among the points from −3 log molarity to distilled water are not statistically significant. The apparent maximum at −5 log molarity may be due to chance variation or uncontrolled chromatographic factors and thus cannot be taken literally. The potency-turbidity curve in Figure 3 indicates that whatever factors are responsible for this behavior, error associated with the nitrogen determination is not the cause, since the same peaking is obtained with an independent procedure. This suggests that some of the fluctuations observed in the nitrogen data may be due to incomplete column control of the faster moving nonvirus components.

The turbidity of each fraction was determined by an abridged spectrophotometer (Aminco, model F), employing a 2-inch cell and a No. 42 filter yielding an effective wave length of 424 mμ. An independent experi-

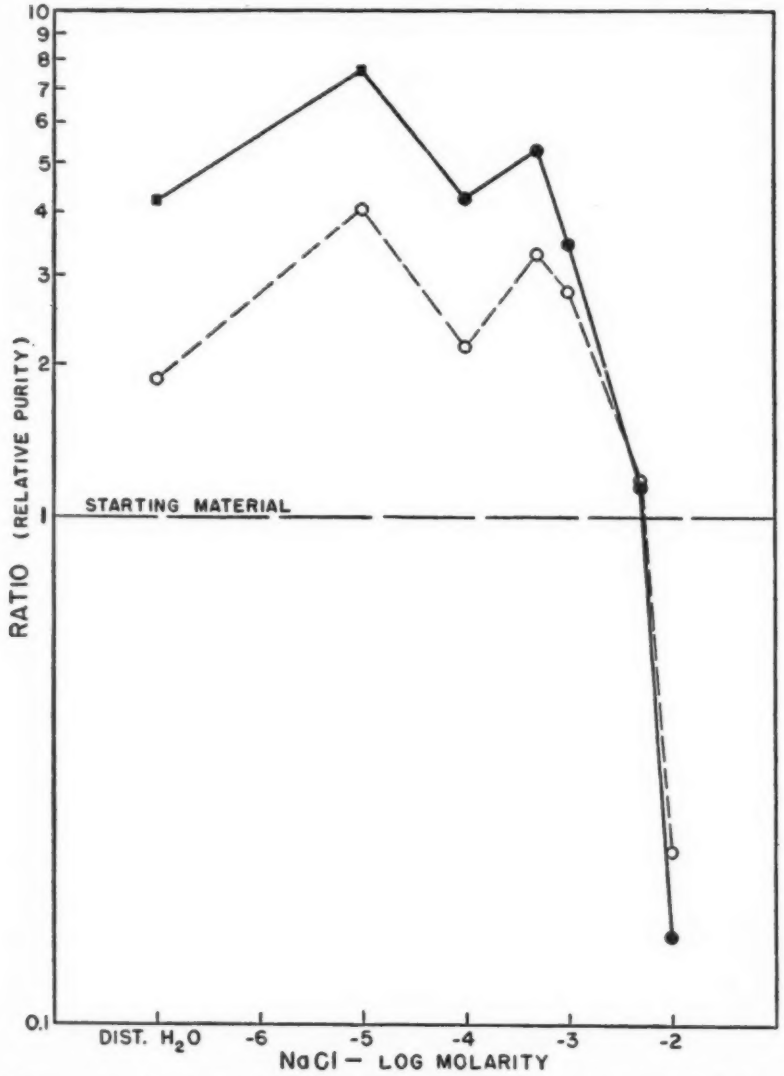
[10] The different location of the apparent peaks in the developed and undeveloped experiments may be a function of the difference in technique employed, though they are not statistically significant. The different potency-nitrogen levels obtained between developed and nondeveloped columns, in the elution range, are highly significant statistically.



TEXT-FIGURE 1.—Effect of salt on the concentration of tumor agent eluted from Celite columns in three independent experiments. Open circles, broken line: Experiment (c), columns not developed. Triangles, solid line: Experiment (d), columns developed. Closed circles, solid line: Experiment (e), columns developed.



TEXT-FIGURE 2.—Effect of salt concentration on the relative purity (based on nitrogen) of the tumor agent eluted from developed and undeveloped Celite columns. Open circles, broken line: Experiment (c). Triangles, solid line: Experiment (d). Closed circles, solid line: Experiment (e).

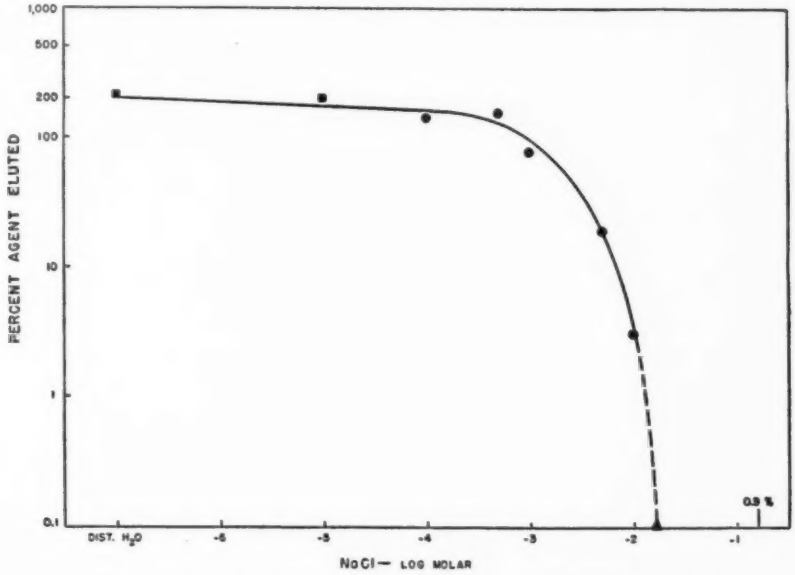


TEXT-FIGURE 3.—Comparison of potency-nitrogen and potency-turbidity ratios resulting from various salt concentrations as eluents. Solid line: Potency-nitrogen ratio. Broken line: Potency-turbidity ratio. Closed circles: Means of experiment (d) and (e). Squares: Experiment (e) only. Open circles: Experiment (e) only.

mental correlation of the increase in turbidity with distilled water as compared with dilute salt solution will be reported in Part II of this series.

In text-figure 4, the percent chicken tumor agent released at various sodium chloride molarities is plotted from the mean of the two comparable quantitative experiments. Potencies and molarities are both plotted as

logs. The resulting curve shows the adsorption, transition, and elution range for the agent irrespective of the release of nonvirus components. Since the curve appeared to be logarithmic, the log potencies were plotted against the actual molarity and a straight line resulted. Either curve may be mathematically transformed[11] to the other with the experimental points falling about the line within the experimental error. This indicates that within the experimental range the log of potency is essentially linear with saline molarity,[12] and that the chicken tumor agent is thus increasingly eluted from the silica column logarithmically with an arithmetic decrease in sodium chloride concentration.



TEXT-FIGURE 4—Elution curve for the chicken tumor agent in the silica-saline chromatographic system.
Circles: Means of experiment (c) and (d);
Squares: Experiment (d) only;
Broken line: Extrapolation from the function $y=a-b\ 10^x$.

DISCUSSION

In this group of experiments, the primary intent of determining the adsorption and elution range of the chicken tumor agent on the silica column with respect to sodium chloride concentration has been accomplished within limits suitable for chromatographic manipulation of the agent. The data from 3 quantitative, and 4 semiquantitative experiments

[11] The equation of the curve shown in text-figure 4 is: $y=a-b\ 10^x$, where y is log potency, x is log molarity, and a and b are equation constants. If the antilog of x, and hence molarity, is designated by z, then $z=10^x$. Direct substitution yields $y=a-bz$ to give the equation for the straight line relationship.

[12] It is possible that the adsorption is a function of ionic concentration rather than molarity though the experimental error does not permit a distinction between the small differences involved.

involving 3,388 chicken test sites show that the agent is adsorbed strongly to the diatomaceous silica (Celite) at the physiological level of 0.154 (and 0.1) molarity; and continues to be adsorbed, with only a slight decrease, to 0.01 molar concentration. The adsorption decreases rapidly from 0.01 to 0.0001 molarity, where the curve levels asymptotically and the percentage adsorption of the agent is no longer a highly significant function of the salt concentration.

Two factors of chromatographic and biologic interest are illustrated by the data. (1) Concentration: From table 3 it is seen that all eluates from −3.3 log molarity to distilled water release more agent per unit-volume than was present in the starting extract. This demonstrates agent accumulation on the column under adsorption conditions, followed by a subsequent enrichment of the agent in the eluate. (2) Purity: Text-figures 2 and 3 illustrate the gain in purity of the agent (from nitrogen and turbidity criteria) in all eluates from −3 log molarity through distilled water. The relative purity, as expressed by the potency-nitrogen ratios, showed an average of 5.2 for the mean of the "plateau" points, and a maximum of 7.6 for the −5 log eluate; or a gain, over the partially purified extract, of 420 and 660 percent, respectively.

It is generally recognized that adsorption phenomena (*33*, *34*, *35*) of some sort, including the influence of pH, effects the passage of viruses through filter candles of the Berkefeld type. Since the reported composition of this filter is compressed diatomaceous earth (*36*), adsorption of some viruses, with consequential filtration failure in the presence of the isotonic saline preparations frequently employed as suspension media, might be expected. This has been reported in the past for the chicken tumor agent but with other assigned reasons (*37*). However, negative filtrates were obtained in the course of these studies with highly active starting material when the salt concentration was 0.9 percent.

Although it is not advisable to extrapolate these findings to other untested viruses without reservation, the data indicate that filtration operations intending to demonstrate the presence of this agent, or similar behaving agents, should be done in a low ionic environment not exceeding 0.001 molarity. The standard isotonic salt solutions such as Lockes', Ringer's, Tyrode's, *etc.*, usually exceed this limit considerably. Failure to demonstrate virus-like agents in various mammalian tumors is probably due to more fundamental difficulties than adsorption on a diatomaceous or other filter candle. However, if an agent were present in relatively small quantities and tended to behave like the chicken tumor virus, filtration failure would be reasonably predicted in the presence of physiological concentrations of salt, even though all other conditions were satisfied.

It has not been determined to what extent nutrient broth and other protein solutions may neutralize the adsorption inducing effects of salt beyond the finding that adsorption of this agent was not noticeably impaired in the presence of a high concentration of the normal proteins of a crude tumor extract in physiological saline.

As an incidental point, these data suggest that the promiscuous use of diatomaceous filter aids, such as Celite, for various plant and animal extracts should be done with the knowledge that they may constitute, in some cases, a short chromatographic column with attendant uncontrolled adsorption, as well as filtration, phenomena.

SUMMARY

The use of chromatographic adsorption principles applied to the problem of separating the virus of chicken tumor I from the closely associated tissue components is reported. The behavior of the agent in respect to adsorption, elution, purification, and recovery in a chromatographic system composed of diatomaceous silica (Celite) and various molar concentrations of sodium chloride is described.

Several statistically controlled quantitative experiments demonstrated strong adsorption of the agent on the column, when the salt concentration was at the physiological level (0.9 percent, 0.154 molar). As the salt concentration was decreased, the elution of the virus increased logarithmically to an asymptotic region between −4 log molarity and distilled water.

Tumor-agent preparations purified by a previously described method were concentrated and further resolved on the Celite column to yield virus-nitrogen ratios over five times the starting material, with a maximum increase of 660 percent virus per unit of nitrogen.

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CHROMATOGRAPHIC STUDIES ON THE SEPARATION OF THE VIRUS FROM CHICKEN TUMOR I. II. ZONING, PURIFICATION, AND RECOVERY OF THE AGENT FROM THE COLUMN[1,2]

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A chromatographic system suitable for the manipulation of the agent of chicken tumor I was described in the preceding paper of this series (*1*) and in a brief report (*2*). Since the conditions for adsorption and elution of the agent without gross denaturation were determined, the primary concern of this group of experiments was to determine whether this virus-like agent and closely associated normal constituents could be made to demonstrate typical chromatographic zoning phenomena, and thus be utilized in purification and characterization studies. The following experiments show the area of concentration for the agent in the silica-saline system, the accompanying segregation of nonvirus nitrogen components, and the resulting purification and total recovery.

MATERIALS AND METHODS

The materials and methods described in detail in the preceding paper (*1*) apply in general to these experiments, with the following exceptions and modifications.

COLUMNS, ADSORBENTS, AND SOLVENTS

A single column, 34 by 230 mm., designed for extrusion of the adsorbent was employed. This was constructed with a sintered glass disk sealed at the end of the male connection of the 34/28 standard taper glass joint.[4]

The adsorbent in all experiments was diatomaceous silica (Celite) of the grades previously described.

Solvents were limited to physiological sodium chloride (0.9 percent) and distilled water.

The rate of flow for adsorption and development of the tumor extracts employing the large column was relatively fast, approximating 10 ml. per minute.

TUMOR AGENT

The virus-like agent of chicken tumor I (Rous sarcoma) (*3*), was used on the columns as a partially purified suspension prepared by two different methods. The "second extraction" procedure has been previously described (*4*) and is termed material B herein. Differential centrifugation

[1] Received for publication, March 15, 1950.

[2] Presented in part at the meetings of the American Association for Cancer Research, Chicago, May 16–17, 1947.

[3] Statistical advice and counsel were provided by Mr. Jerome Cornfield, and statistical computations in the analysis of variance of the bioassays were performed by Miss Virginia Voorhees.

[4] Chromatographic Tube, size III, Scientific Glass Apparatus Company, Bloomfield, N. J.

technique, essentially the same as described by Claude (*5*), was applied to 10 gm. of thawed tumor tissue. The final pellets were resuspended in 30 ml. of 0.005 molar phosphate buffer, pH 7.0, and cleared at 18,000 times gravity for 1 minute,[5] (plus de-acceleration time), to remove aggregates. The resulting opalescent solution is referred to as material A.[6]

EXPERIMENTAL PROCEDURES AND RESULTS

(*a*) *Zoning of agent and nitrogen from centrifugally purified material.*— In the first experiment the tumor agent was prepared by differential centrifugation as described for material A under Materials and Methods, with the exception that prior to final volume adjustment concentrated sodium chloride was added to give a molar concentration of 0.154 or 0.9 percent. Two batches were processed to provide 60 ml. of concentrated agent.

The chromatographic column was prepared by placing a circle of Whatman No. 1 filter paper on the sintered glass disk at the bottom of the large 34 mm. column, and with a slight vacuum applied to the filtering flask, a suspension of adsorbent consisting of 10 gm. of No. 501 Celite suspended in 200 ml. of distilled water was added. Following sedimentation, the column was further washed with 200 ml. of distilled water, followed by 100 ml. of cold 0.154 molar sodium chloride solution. This yielded a column 38 mm. high.

Fifty milliliters of cold tumor-agent preparation were added gradually, with precautions taken to prevent the column from going dry at any time during the process. The column was then developed with 100 ml. of cold 0.154 molar sodium chloride solution. The vacuum was continued a few seconds following the removal of the last of the saline, and the entire adsorbent column was extruded from the glass tube, and bisected longitudinally. One longitudinal section was employed for staining correlations,[7] while the other half was segmented into four portions consisting of:

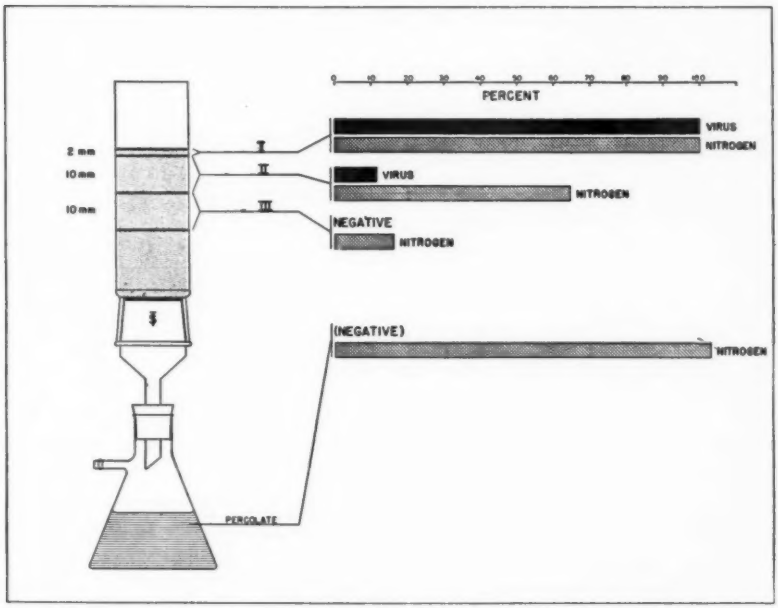
I, a thin 2-mm. segment from the top of the column; II, a 10-mm. section immediately following; III, a second 10-mm. section; and IV, the remainder of the column which was not tested in this experiment. The first three segments represented the unknowns, and were treated as follows: Each was placed in a small beaker and suspended in 10 ml. of eluent consisting of cold distilled water. These suspensions were each poured on a No. 1 filter paper in a small Buchner funnel attached to a

[5] Multispeed attachment for International Centrifuge No. 2, head No. 295, 6 place, used with 13 ml. collodion tubes.

[6] Modification of Claude's technique was introduced in the preparation of this material as follows: It was found that much of the muscle and connective tissue associated with breast grown tumors could be eliminated by passage in the ventral surface of the wing. Clearance by centrifugation was substituted for Berkefeld filtration, the concentration time in the centrifuge was reduced to 1 hour per cycle; and 0.9 percent saline and phosphate buffer were employed as solvents.

[7] Since the partially purified chicken tumor agent is colorless on the chromatographic column, no visible zones are available to guide the sectioning. In an attempt to circumvent this difficulty some preliminary work has been done to make the bands visible by spraying the extruded column, or a portion thereof, with a dilute dye solution. Two dyes, phloxine and toluidine blue, have revealed zoning characteristics on the extruded adsorbent. Segmentation of the column in this experiment was done partially from this staining reaction and partially from empirical observations of other experiments. Since insufficient data or knowledge is available to establish correlation between colored zones and agent, no attempt is made to describe the procedure in this report. It is not known whether the stained zone at the top of the column is the virus or a closely associated substance.

sterile 50 ml. filtering flask under vacuum. As soon as the adsorbent was sucked dry, it was washed with a vigorous stream from a pipette until a total volume of 25 ml. of eluent had been employed for each segment. This represented the starting volume of the original agent for the longitudinal half column. Each of these unknown eluates was tested quantitatively for tumor-agent content and for total nitrogen. The percolate was analyzed for nitrogen only.[8] The results are shown in table 1 and in text-figure 1.



TEXT-FIGURE 1.—Separation of centrifugally purified chicken tumor virus and contaminating nitrogenous substance with the Celite column.

(*b*) *Zoning of agent and nitrogen from "second extraction" purified material.*—The secord experiment was an elaboration of the one just described but differed in some details. The source of agent for the column was partially purified by the "second extraction" procedure, as described for process (B) in materials and methods, rather than by differential centrifugation. A total volume of 100 ml. of this extract was poured on a column prepared as in the first experiment, except that 10 gm. of Celite No. 503 rather than No. 501 was employed. Following adsorption, the column was developed with 50 ml. of 0.154 molar saline, and the adsorbent column was extruded as before. Five segments of the column were separated in this experiment and tested quantitatively for tumor agent and

[8] The filtrate was not tested for percent agent in this experiment but was found negative in previous experiments, and less than 1 percent in a subsequent quantitative experiment.

TABLE 1.—*Relative percent tumor agent and nitrogen recovered from different regions of the chromatographic column and in the filtrate when starting with an agent partially purified by differential centrifugation*

Reference	Determination	Starting material	Zone I eluate	Zone II eluate	Zone III eluate	Percolate [1]
Starting material A	Nitrogen, mg./ml.	0. 135	0.0093	0. 0060	0. 0015	0. 0096
	Nitrogen, percent	100. 0	6. 9	4. 4	1. 1	7. 1
	Potency, percent	100. 0	18. 8	2. 2	Negative	Negative [2]
	Agent recovery, percent.	(100. 0)	18. 8	2. 2	0	0
	Standard error of potency estimate.		+9. 6 −6. 3	+5. 8 −1. 6		
	Potency-nitrogen ratio.	1. 0	2. 7	. 5		
Zone I [3]	Nitrogen, percent		100. 0	64. 4	16. 1	103. 2
	Potency, percent		100. 0	11. 5	Negative	Negative

[1] Calculations based on 50 ml. volume.
[2] See text footnote 8.
[3] Reference employed in text-figure 1.

nitrogen: a thin 2.5-mm. section from the top of the column, the two following 5-mm. segments, the next 10-mm. segment, and the remaining 20 mm. of adsorbent were deposited in small beakers and suspended in 10 ml. of cold distilled water with the aid of a small stirring rod. These suspensions were then each poured on a No. 1 Whatman filter paper in a small Buchner funnel and washed with a vigorous stream of cold distilled water from a 10-ml. pipette. Forty milliliters were employed for the washing process, making a total of 50 ml. of eluent, which equaled one-half the starting volume.

The results are shown in tables 2 and 3 and text-figures 2–5.

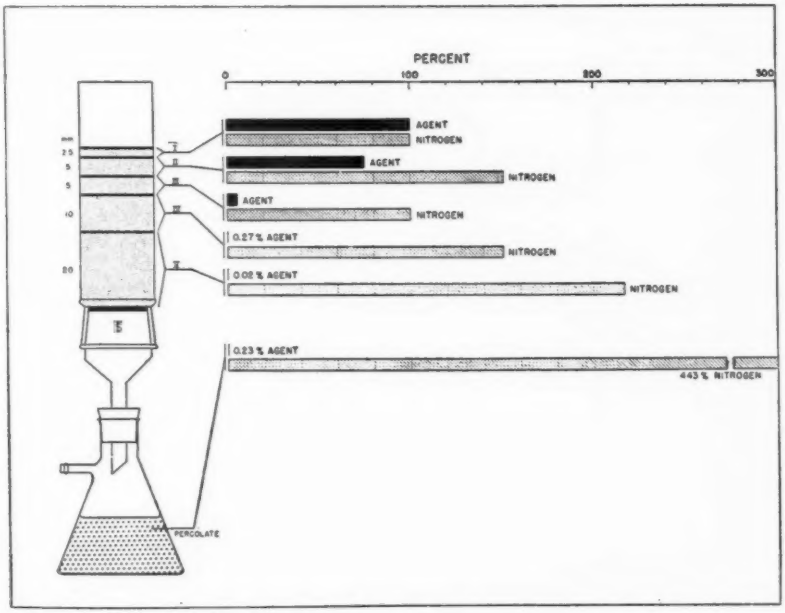
Graphic illustration of the concentration region of the virus under the described conditions of the two experiments is shown in text-figures 1 and 2. In both instances the highest concentration of the tumor agent is in the top 2 or 3 mm. of the column, with a rapid decrease in the descending zones. In text-figure 3 the rate of decrease of the agent concentration related to the distance from the top of the column is shown on a log scale which permits the spatial plotting of the low agent values. This decrease in concentration down the column, with the exception of the highly concentrated upper zone, is approximately logarithmic.

In contrast to the regional concentration of the tumor agent, the figures show that nitrogen, unassociated with virus activity, is distributed throughout the column and in the filtrate. In experiment (b) the total nitrogen found in the lowest segment was more than twice that eluted from the top zone, while the virus recovered was less than one thousandth of the activity in the top zone eluate. A similar relationship may be noted in the percolate and in the intermediate zones. This virus-nitrogen relationship for the various portions of the column and the filtrate is shown by the potency-nitrogen ratio data in table 2 and text-figure 4.

In experiment (b) the top zone eluate contained a virus concentration of 52.2 percent of the activity of the partially purified starting material,

while it contained but 8.1 percent of its nitrogen. Since the eluate volume was one half the adsorbed volume, the total recovery of virus from this zone was 26.1 percent of the total starting virus and 4.05 percent of the starting nitrogen. Thus, 26 percent of the virus was recovered from the 2.5 mm. top zone in a state of purity 6.45 times that of the starting material, or with a 545 percent increase in virus per unit of nitrogen.

Text-figure 3 shows a drop of agent concentration of more than twenty-fold in the first 10 mm. of the column, whereas the total nitrogen decrease is less than twofold in the same distance. This is consistent with the other data and the chromatographic concept of retaining a given substance in a

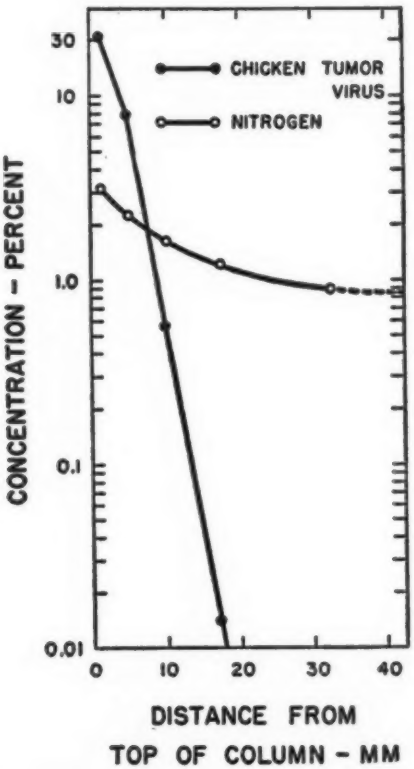


TEXT-FIGURE 2.—Resolution of virus and contaminating nitrogenous material on the Celite column following partial purification by extraction.

relative stationary position while distributing the other components throughout the column and into the percolate by development. Although no chemical studies other than nitrogen and Biuret tests have been made of the components in the other regions of the column, some of them presumably are proteins. Suggestive, but inconclusive, evidence that normal particulates are present comes from unpublished electron-microscope studies of the eluates from the various segments of the column. Particles were seen in high population in the lower segments, where there was essentially no virus activity, and were morphologically of the same category as the particles observed in the upper segments where the tumor agent was concentrated. Various interpretations are possible, but the

simplest, from the chromatographic viewpoint, is that there has been a segregation of virus and normal particles on the column.

The data from these two quantitative experiments indicate that the particulates associated with the tumor-producing principle of chicken tumor I behave in an acceptable chromatographic fashion on the diatomaceous silica column when employing 0.154 molar sodium chloride and distilled water as solvents. As a consequence of this behavior, a partitioning



TEXT-FIGURE 3.—Selective distribution of tumor agent and nitrogen on the Celite column. (Semi-log plot. 1.0 mm. equals 0.235 gm. of Celite (#503), 1.0 gm. equals 4.25 mm. of column adsorbent.)

occurs between the virus and nonvirus nitrogen components of the starting extract. The significance of this resolution in terms of absolute purity of the agent is uncertain at this stage and awaits further investigations.

(*c*) *Purification, concentration, and recovery of the chicken tumor virus from crude tumor extract.*—All experiments so far reported have employed as a starting material an agent preparation partially purified by extraction or differential centrifugation. This experiment was intended to check the effectiveness and efficiency of the silica column in segregating the agent

TABLE 2.—*Relative percent virus and nitrogen concentration recovered from different regions of the chromatographic column and in the filtrate when starting with an agent partially purified by second extraction*

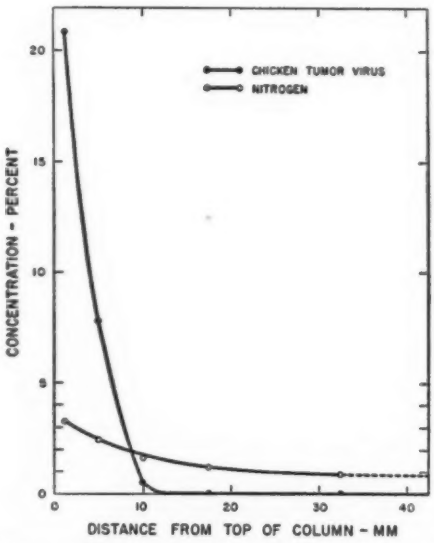
Reference	Determination	Starting material	Zone I eluate	Zone II eluate	Zone III eluate	Zone IV eluate	Zone V eluate	Percolate [1]
Starting material B	Nitrogen, percent	100. 0	8. 1	12. 2	8. 1	12. 2	17. 6	35. 9
	Potency, percent (virus/ml.)	100. 0	52. 2	39. 1	2. 8	. 14	. 01	. 12
	Virus recovered, percent	(100. 0)	26. 1	19. 6	1. 4	. 07	. 005	. 38
	Standard error of potency estimate	----------	+25. 1 −17. 0	+21. 1 −13. 7	+4. 6 −1. 7	+. 3 −. 1	----------	+. 3 −. 1
	Potency-nitrogen ratio	1. 0	6. 4	3. 2	. 35	. 01	. 0006	. 003
Zone 1 [2]	Nitrogen, percent	----------	100. 0	151. 0	100. 0	151. 0	217. 0	443. 0
	Potency, percent	----------	100. 0	74. 9	5. 4	. 27	. 02	. 23

[1] Calculations based on 100 ml. volume.

[2] Reference for text-fig. 2.

TABLE 3.—*Distribution and recovery of total nitrogen and chicken tumor agent from a segmented column*

Fraction	Nitrogen concentration mg./ml.	Volume, ml.	Total nitrogen mg.	Total nitrogen recovered percent	Total virus recovered percent
Starting material	0. 0074	100. 0	0. 740	100. 0	(100. 0)
Zone I eluate	0. 0006	50. 0	. 030	4. 1	26. 1
Zone II eluate	. 0009	50. 0	. 045	6. 1	19. 6
Zone III eluate	. 0006	50. 0	. 030	4. 1	1. 4
Zone IV eluate	. 0009	50. 0	. 045	6. 1	. 07
Zone V eluate	. 0013	50. 0	. 065	8. 8	. 005
Total recovered from column		250. 0	. 215	29. 2	47. 2
Percolate	. 0016	166. 0	. 266	35. 9	. 38
Total recovery		416. 0	. 481	65. 1	47. 6



TEXT-FIGURE 4.—Concentration of virus and nitrogen per mm. of column adsorbent. 1.0 mm. equals 0.235 gm. of adsorbent.

from a crude extract as compared with its partitioning of partially purified material.

The column was prepared as in experiment (b). A 10 percent tumor extract was prepared in the usual manner by homogenizing 10 gm. of chicken tumor in the blender for 1 minute with 70 ml. of 0.9 percent saline solution, and another minute with a total of 100 ml. This extract was cleared twice in the multispeed centrifuge at 10,000 r. p. m. (approximately 6,000 times gravity). The first run was 5 minutes and the

second run 10 minutes. This centrifugation deposited most of the tissue debris and yielded a typical reddish, slightly turbid, "first extract." The volume was adjusted to 100 ml. with 0.9 percent saline, and the solution was adsorbed on the column.

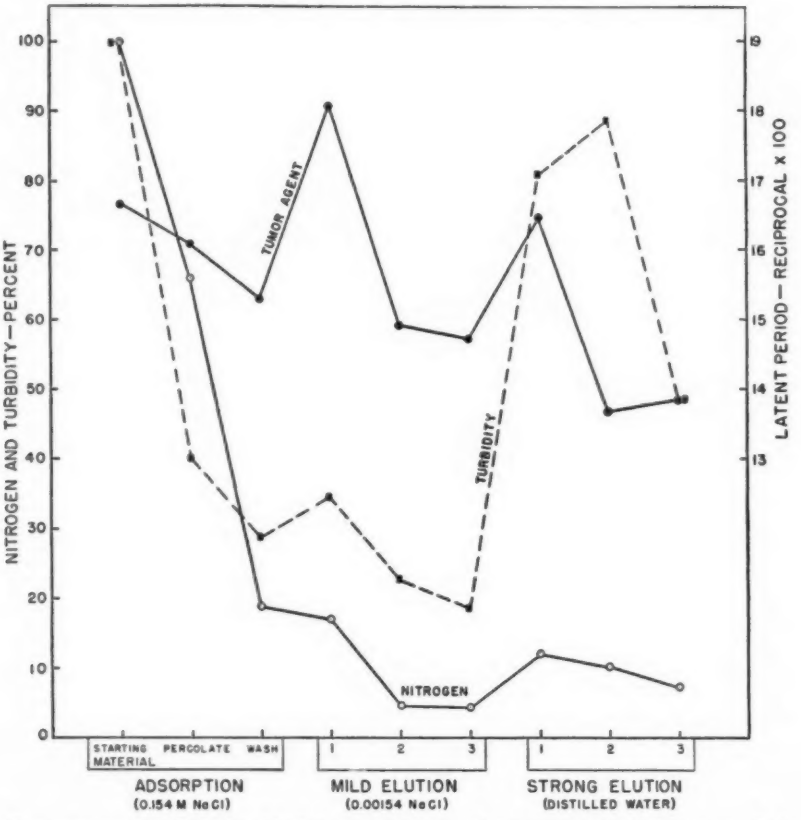
The reddish colored components migrated down the column and into the filtrate during the percolation process, and were completely removed by the 100 ml. of 0.9 percent saline used for development. The extruded column was white except for a 2 mm. amber zone at the top. The top 5 mm. of the column was severed and resuspended in a small beaker with 10 ml. of distilled water and left in the refrigerator for approximately 10 minutes. The suspension was eluted in the usual way by pouring it onto a No. 1 (4.25 cm.) Whatman filter disk in a small Buchner funnel and washing the adsorbent with an additional 20 ml. of cold triple-distilled water. This eluate was tested quantitatively against the starting extract for potency and nitrogen. The bioassay was run in duplicate for this fraction, using two separate comparisons with different dilutions. Good potency agreement was obtained at 86.7 and 72.3 percent virus concentration compared to the starting extract.

The distilled water eluate from the top 5 mm. of the column contained a mean of 79.5 percent concentration of the activity of the original extract and 3.9 percent concentration of the nitrogen. Thus, 24 percent of the total virus contained in 100 ml. of crude extract was recovered in 30 ml. of eluent from the top 5 mm. zone of the column at a virus concentration of approximately 80 percent of the original extract. The total nitrogen in this fraction was 1.1 percent of the original, which gave a purification of 20.4 times the starting material in a single operation.

If a comparison of the relative purification obtained, based on nitrogen, between this experiment and experiment (b) is made, it will be noted that the final potency-nitrogen ratio of the eluate, from the one-stage procedure using the chromatographic column only, was about the same or slightly higher than that obtained when the agent was first partially purified by an auxiliary method. This does not rule out the possibility that the two-stage procedure is superior when a criterion other than nitrogen is used.

(*d*) *Abridged effluent curve of virus, nitrogen, and turbidity.*—Text-figure 5 illustrates the approximate relationship between the tumor agent, nitrogen, and turbidity when a single column is successively developed and eluted with a mild eluent followed by comparable elution with a strong eluent.

Three semiquantitative experiments performed in essentially the same way and yielding the same general results are illustrated by plotting their means as shown in text-figure 5. A microcolumn was prepared, in the usual way, from a slurry of No. 503 Celite, which yielded a column 8 x 35 mm. containing 0.4 gm. of adsorbent. The small column was intentionally overloaded by adding gradually 100 ml. of extract B. The 100 ml. of percolate was saved for testing, and the receiving flask was changed with each successive 10 ml. of solution. The column was first eluted with 0.00154 molar saline, and in all but one experiment three successive



TEXT-FIGURE 5.—Relationship between potency, nitrogen, and turbidity, on a single column subjected to successive elutions.

10-ml. portions were employed. The column was then subjected to the additional and more rigorous elution of distilled water. In all experiments the column was eluted at least once with this solvent, and in one experiment it was eluted with three successive 10-ml. portions.

Each fraction, including the starting material, was tested for approximate activity employing a total of 232 chicken sites. The latent period of each site response was converted to its reciprocal, and averaged for each fraction. The solid line in text-figure 5 represents the mean of the 3 experiments. The virus potency difference between fractions in any one experiment probably could not be considered statistically significant; however, since the 3 separate experiments maintained a similar relationship between fractions, the composite data provide a reasonably reliable ordinal relationship for the conditions described.

The turbidity of each fraction was determined by an abridged spectrophotometer (Aminco, type F) employing a 2-inch cell and a No. 42 filter yielding an effective wave length of 424 mμ.

Nitrogen determinations were made as in previous experiments. The points of the various fractions are plotted in chronological order and are connected by lines for convenience in comparing the relationship between potency, nitrogen, and turbidity and to indicate the abridged effluent curve. A virus optimum was obtained in each experiment with the first 10 ml. of 0.0015 molar saline, while the nitrogen and turbidity both remained low. There was a decrease in agent with successive elutions of this solvent, and the nitrogen and turbidity also dropped off slightly. From the appearance of the curve at this point, it would appear that most of the agent had been removed with the first elution; however, treatment of the column with distilled water yielded a secondary potency peak, a slight increase in nitrogen, and a substantial increase in turbidity. The turbidity was not expressed in the previous elution. Successive treatment with this solvent failed to elicit further high concentrations of the agent, whereas the turbidity remained above its previous level. This experiment demonstrates the advantage of a moderate eluent over distilled water from the standpoint of nonnitrogenous impurities.

DISCUSSION

The separations and characterizations of ordinary molecules have been accomplished largely through their capacity to undergo the rigorous procedures of fractional distillation, extremes of pH, and exposure to a wide variety of solvents and conditions without losing their identity. Unfortunately, this type of attack is not suitable for the purification and characterization of the infinite varieties of labile macromolecules and particulates of cellular origin. The centrifuge has been the most successful tool applied to the problem, partly because its denaturation of particles is restricted. Its separation limitations are nevertheless evident and have been discussed and analyzed (*6–8*).

The phenomena of chromatography and its remarkable separations of closely related compounds, such as the amino acids (*9*), raise the question whether this capacity for separation of substances with but slight differences can be applied to the general field of macromolecules and particulates, of which viruses, bacteriophage, microsomes, mitochondria, *etc.*, are composed. This report of the chromatographic behavior of one virus partially answers a portion of the question. It indicates that particles of this magnitude do respond to the forces of adsorption in a manner necessary for typical chromatographic manipulation, and that it can be accomplished in a relatively inert system where the degree of denaturation is within reasonable limits. Of the several countercurrent procedures that have been so successfully used in the separation of molecules, chromatography appears to be the only one that lends itself to the gentleness necessary for labile subcellular particulates.

In these studies little use has been made of the centrifuge except for preparation of starting material. The "short column procedure" (*10*) demonstrates that even this limited use of the centrifuge can be dispensed with in the preparation of some cell-free cytoplasmic extracts. However,

for ultimate purifications the concomitant use of chromatography and differential centrifugation may provide in some instances a combination superior to either alone.

While emphasis in these reports has been directed to the separation of biological particulates as such, they are, of course, protein complexes existing in colloidal form and appear to behave chromatographically in a general way as do the smaller proteins (*11*, *12*). A test for the detection of tobacco-mosaic virus protein in plant juice, employing paper chromatography, has also recently been reported (*13*). In the investigations so far, no defined effort has been made to determine the mechanism involved in the adsorption of these large protein particles. The results are consistent, however, with the "salting out" phenomena (*14*) recently described for small proteins when the salt concentration is increased in the presence of a suitable adsorbent. This should not be confused with the end-point expression of aggregation and precipitation.

Since this work was done, the same general system has been applied to the separation of some of the particulates of mammalian tumors (*15*). Various enzyme systems were substituted for infectivity as criteria of biological activity of the particles separated. The melanin granules, which were the object of the study, were of a different size magnitude than the chicken tumor virus and possessed quite different physiological characteristics. While this has demonstrated that chromatography can be successfully applied to particulates of considerable size and physiological variation, the scope of the experience so far permits only limited extrapolation of the ultimate applicability of the method.

SUMMARY

The chromatographic zoning characteristics of the virus-like agent of chicken tumor I on diatomaceous silica (Celite) columns are described. Resolution of agent and nonactive nitrogen components are demonstrated.

Tumor agent, purified by previously described methods, was further separated from contaminating nitrogenous components to yield a maximum potency-nitrogen ratio of 6.4 times the partially purified starting material.

Approximately 24 percent of the virus from 100 ml. of a crude tumor extract was recovered in a single fraction at a concentration of approximately 80 percent of the starting preparation. The potency-nitrogen ratio indicated a twentyfold increase in agent purification in a single stage operation.

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CHROMATOGRAPHIC STUDIES ON THE SEPARATION OF THE VIRUS FROM CHICKEN TUMOR I. III. TITRATION CURVE OF THE AGENT [1]

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The potency and degree of purity of virus preparations are frequently indicated by the end-point titration and expressed as nitrogen or dry weight per minimal infective dose. While the accuracy of this procedure is limited because of its dependency upon number of sites and animal variation as well as experimental error, the following quantitative experiment contains relatively good internal consistency with respect to the relationship of the titration curve, tumors, and nitrogen. The approximate end point of the chromatographed chicken tumor agent is therefore reported, along with other findings, for comparison with previously published data employing these criteria (*1*, *2*).

The 50-percent-infectivity point (ID_{50}) is probably a more stable and satisfactory reference for expressing nitrogen and virus concentration in comparisons between various methods, experiments, and agents. The slope of the sigmoid titration curve is much steeper in the mid-region than at either end, and is thus intrinsically more accurate at this point. In addition, the data of the entire curve are employed in determining the 50-percent point, whereas the exact end point may be determined by the chance presence or absence of a single tumor, unless a linear function is known or assumed and the probable "end point" determined by extrapolation. However, the 50-percent point by itself does not give a satisfactory indication of the minimal number of particles required for infection, since it is uncertain whether an extrapolated probit function is valid at the extreme ends of the titration curve.[3] Therefore, the experimental "end point" is employed in the discussion of the minimal infective dose.

EXPERIMENTAL PROCEDURES AND RESULTS

Materials and methods, other than those described below, were the same as have been reported in detail in preceding papers (*3*, *4*, *5*).

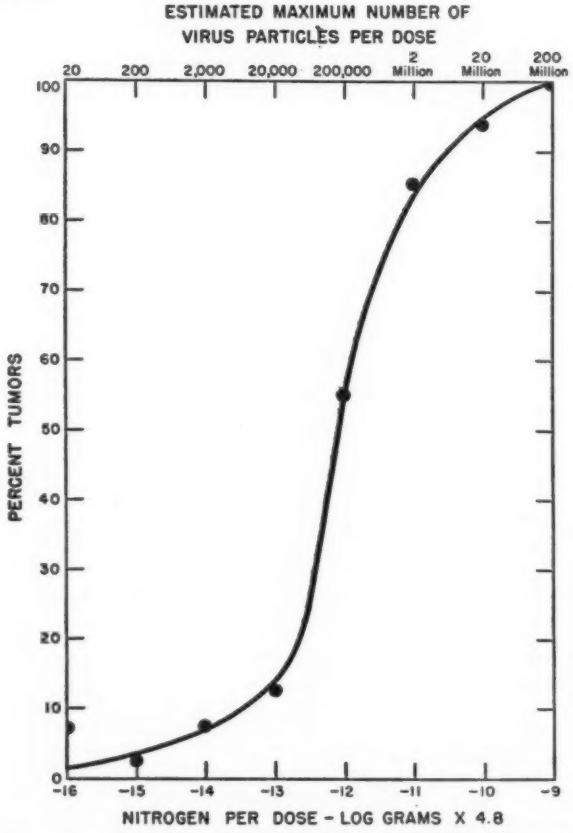
An agent preparation that had been through the Celite column (*3*) was titrated in chickens at tenfold dilutions from 10^{-3} through 10^{-10}. The nitrogen concentration at the zero dilution was 2.4×10^{-2} mg. per ml., or

[1] Received for publication, March 15, 1950.

[2] Statistical counsel provided by Jerome Cornfield.

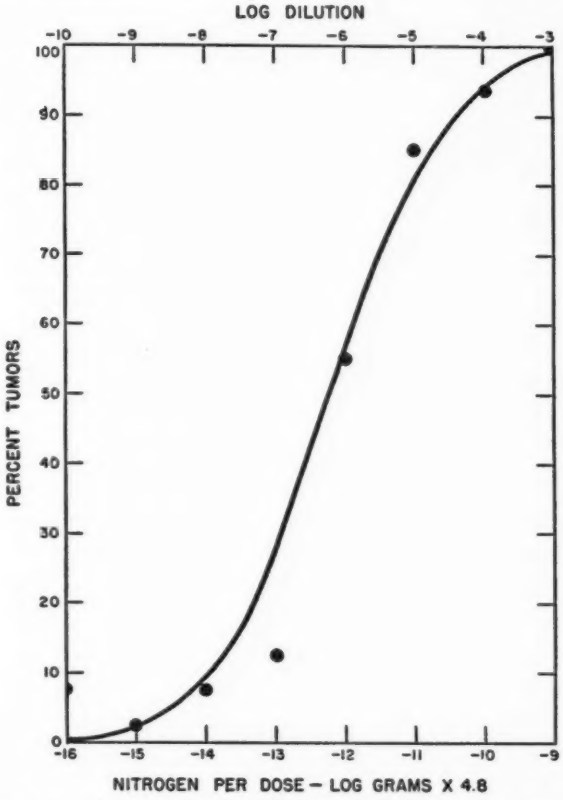
[3] In this experiment, if the last point of the titration curve is discarded the probit function describes the data within the expected limits of variation. More extreme deviations from the fitted curve than those obtained would be expected by chance about 1 time in 5, ($\chi^2=3.1$, d. f.$=2$). If the last point is retained, and a curve fitted to the data as shown in text-figures 2 and 3, the deviation from the probit function at the bottom of the curve is greater than would be expected by chance variation.

4.8×10^{-6} gm. per dose (0.2 ml.). Each dilution was inoculated at 40 sites except at 10^{-3} and 10^{-4}, where there were 32 sites. The total tumors induced by the various dilutions, and other data, are listed in table 1, and the resulting titration curve is shown in text-figures 1, 2, and 3. Since tumors were obtained at all dilutions, though in decreasing numbers, an absolute negative end point was not realized, although the nitrogen of the titration curve indicated a relatively close approach to the single particle concentration.



TEXT-FIGURE 1.—Titration curve of a chromatographically purified chicken-tumor-virus preparation. Line drawn through the experimental points by inspection.

Since the dilution technique is critical in an experiment of this type, special attention was paid to volumes, mixing, and prevention of cross contamination. No volume of less than 1 ml. was measured in the preparation of the dilution series, and with the exception of 10^{-1} and 10^{-2}, which were not used for inoculation, 2 ml. of serum-buffer and virus mixture were added to 18 ml. of sterile serum-buffer to yield the decreasing tenfold virus dilutions. A fresh pipette was employed for each dilution,



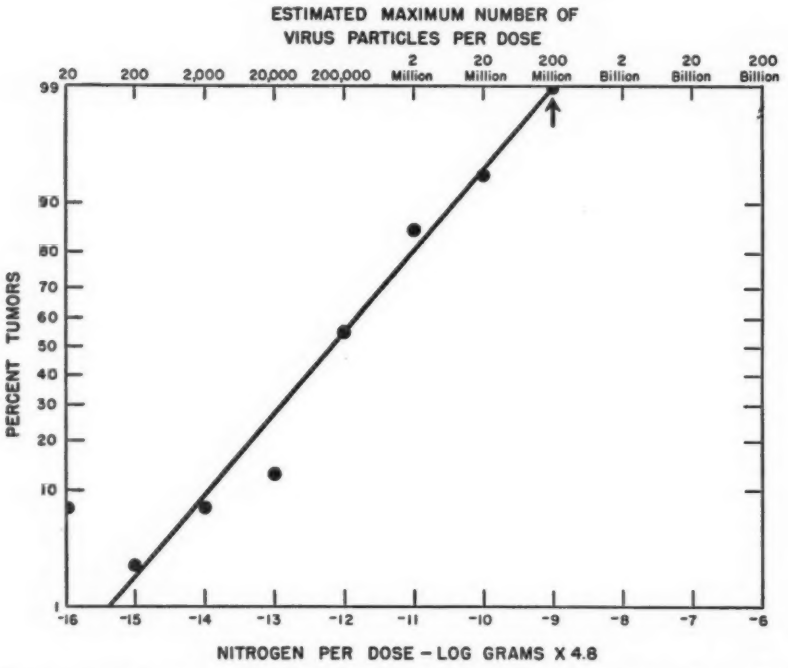
TEXT-FIGURE 2.—Probit curve fitted to the titration data. All experimental points employed. (See footnote 3.)

TABLE 1.—*The nitrogen and tumor response relationship of a chromatographically purified chicken tumor virus titrated logarithmically in chickens*

Log dilution	Nitrogen mg./ml.	Nitrogen per dose grams/0.2 ml	Number of chicken sites inoculated	Total tumors	Tumors percent	Estimated percent tumors from probit function	Mean latent period of responding sites (days)	Estimated maximum virus particles per ml.[1]
0	2.4×10^{-2}	4.8×10^{-6}	0					10^{12}
−3	2.4×10^{-5}	4.8×10^{-9}	32	32	100.0	98.9	7.45	10^{9}
−4	2.4×10^{-6}	4.8×10^{-10}	32	30	93.7	94.2	7.45	10^{8}
−5	2.4×10^{-7}	4.8×10^{-11}	40	34	85.0	80.2	10.16	10^{7}
−6	2.4×10^{-8}	4.8×10^{-12}	40	22	55.0	55.2	12.36	10^{6}
−7	2.4×10^{-9}	4.8×10^{-13}	40	5	12.5	27.8	13.60	10^{5}
−8	2.4×10^{-10}	4.8×10^{-14}	40	3	7.5	9.5	14.00	10^{4}
−9	2.4×10^{-11}	4.8×10^{-15}	40	1	2.5	2.1	19.00	10^{3}
−10	2.4×10^{-12}	[2] 4.8×10^{-16}	40	3	7.5	0.3	17.00	10^{2}

[1] For possible sources of error in this estimate see text footnotes 4 and 5.

[2] Pirie (*1*) reported a minimal infective dose of 10^{-4} gm. of nitrogen with an eluate from flask adsorption studies, and Claude (*8*) reported the smallest dose to produce tumors with the centrifugally purified material was about 4×10^{-12} in terms of dry weight or about 4×10^{-14} gm. of nitrogen.



TEXT-FIGURE 3.—Linear probit function fitted to all experimental points. (See footnote 3.)

and the chicken inoculations were made from stoppered serum bottles, each with its own syringe. The preparation of this material (*3*) was such that the presence of virus aggregates was reduced to a minimum, since differential centrifugation (*2*) plus passage through Celite columns would tend to eliminate any aggregates that might have been present.

The nitrogen determination of the zero dilution was run in duplicate with a disagreement of less than 0.005 of a milligram, or 15 percent. The turbidity of the virus preparation recovered from the column was determined with a spectrophotometer at an effective wave length of 424 mμ, using a 2-inch cuvette. The light transmission was 30 percent, the optical density approximately 0.52.

DISCUSSION

From filtration and sedimentation studies many of the physical and chemical properties of the chicken tumor agent have been reported (*2*, *6*). Employing the particle weight of 2.2×10^{-16} gm. and the nitrogen content of about 9 percent, the approximate nitrogen of a single particle can be estimated and compared with the nitrogen content of the various dilutions in this experiment of which the last dilution, the 50-percent point, the −3 log dilution, and the starting material are the most interesting. It is seen that tumors were still obtained at the −10 log dilution,

which contained a calculated nitrogen concentration of 4.8×10^{-16} gm. per dose. A single chicken-tumor-virus particle of 70 mμ diameter is calculated to contain approximately 0.2×10^{-16} gm. (*2*), which implies that if the virus preparation was pure and all of the nitrogen was virus nitrogen, a maximum of 24 particles on the average would be expected in each 0.2-ml. dose at the −10 log dilution. It is not assumed that the virus preparation was entirely pure,[4] therefore this number should represent a maximum if no experimental errors are involved, and is reduced in proportion to the amount of nonvirus contaminating nitrogen present in the chromatographic fraction.[5] This estimate is not dependent upon the biological data, except in so far as the presence of tumors indicates the presence of virus and thus the minimum infective dose.

If the reported nitrogen percent in the virus protein used in the above calculations is too low, owing to the presence of lipoids or other low nitrogen-containing (*2*) impurities in the centrifugally purified material, and a value not of 9 but of 15 percent is assumed as reported for rabbit papilloma (*7*), the maximal number of particles at −10 log dilution is reduced from 24 to 15. While it is not possible, from this experiment, to determine the absolute number of virus particles necessary to induce a tumor in a susceptible chicken, these data suggest that a relatively small number of the order of 1 to 100 can initiate the process. This represents a substantial reduction in the previously reported estimate of 2,000 as a minimal infective dose (*2*).[6]

The nitrogen content of the dose level at the 50-percent point (*8*) in this experiment was $10^{-11.42}$ gm., which if all were virus nitrogen would account for approximately 200,000 particles of 70mμ diameter. Since absolute purity of the preparation is not assumed, this should represent the maximal number of virus particles required for the 50-percent point under the conditions of the experiment. It is of interest to compare these data with that reported for the rabbit papilloma agent (*7*), which is considered to be one of the more highly purified animal viruses. The protein content of the 50-percent infectivity dose is reported to be $10^{-8.355}$ gm. per 0.1 ml. inoculum, or $10^{-9.179}$ gm. of nitrogen, representing 94,000,000 virus particles. This is a marked difference in response between the two tumor-inducing biological systems.

It is seen that with the chicken tumor agent 100 percent tumors were obtained at −3 log dilution with a nitrogen concentration of 4.8×10^{-9} and an estimated maximum of 200 million virus particles per dose. The linear probit function showed an intercept at about 99 percent at −3 log dilution, with an increasing approach to 100 percent as the virus concentration was increased. Although dilutions more concentrated than this

[4] The chromatographically purified preparation employed in this titration experiment represents the product obtained in the early stages of the investigations. While later material has not been titrated to the end point, the lower nitrogen concentration of some of the tested fractions suggested a purification increase over the preparation reported here.

[5] If the size of the agent particle is smaller than the published value of 70 mμ, the maximum number is increased accordingly.

[6] It is possible that a difference in assay technique may have contributed some to the reduction in minimal infective dose since the chance of obtaining tumors at high dilution is partly a function of number of sites or animals injected.

were not inoculated, it is unlikely that with tenfold-increasing doses the response would have been much below the 100-percent point for −2, −1, and 0 log dilutions. While, occasionally, chickens with an abnormally high resistance to the agent are observed, it would appear that the dose at −3 log dilution represents the approximate threshold of virus concentration that overcomes the highest chicken resistance generally found in the population employed.

It might be noted that these data show that the systematically decreasing number of tumors, expressed by the titration curve, is not explained by the chance presence or absence of a virus particle in the inoculum, conforming to the sigmoid curve obtained by the Poisson series and reported as an explanation of the titration curve for another virus (*9*). If Poisson distribution were the case in this experiment, when a tumor was obtained at −10 log dilution 100 percent would be expected at −7 log dilution, whereas less than 20 percent tumors were obtained. Having 100 percent takes at −3 log dilution, 0 percent would be expected at −6 log dilution while 55 percent actually were obtained. This confirms a similar finding for the agent of rabbit papilloma (*7*). While Poisson distribution of virus particles in the inocula at the extreme bottom of the curve could be partially operating, the data indicate that some variety of host resistance is the simplest explanation of the titration curve.

Many of the chromatographic fractions were examined in the electron microscope, including the material described in this experiment.[7] As is the usual case with virus preparations, there was considerable variation between fractions and between microscopic fields of the same fraction. The primary disturbing feature of the electron microscopy, however, was the preponderance of particles considerably smaller than the 70 mμ diameter value reported in the literature (*2*, *6*). The chromatographic fractions with the highest activity yielded particles ranging in size from about 20 to 70 mμ, but with the greatest part of the population grouped about a mean of approximately 40 mμ. This suggests two main possibilities: the purification, while being considerably extended, is either far from complete; or, the chromatographic column in its purification process has stripped a nonessential coating material from the virus that may have given it an enhanced size in the centrifugation and filtration studies. Some support of this possibility is contained in the chemical determinations of the centrifugally purified chicken tumor agent which yielded a relatively large lipoid fraction (35 percent) when extracted with fat solvents (*2*).[8] Since many of the highly purified viruses have been largely protein, with little or no lipoid associated with them, it may be only a tightly bound contaminant in the case of the chicken tumor agent.

SUMMARY

The tumor-response curve resulting from the logarithmic titration of a chromatographed virus preparation in chickens is described. The impli-

[7] With Dr. Herbert Kahler and Mr. Bolivar J. Lloyd.

[8] The chromatographed virus is also more labile than the cruder preparations, which suggests that a protective coating may have been removed.

cations of the curve in respect to an estimate of the number of virus particles in the fractions, the minimum number required to induce a tumor, the number required at the 50-percent-infectivity point, and other questions are discussed, based on the determined nitrogen of the preparation and the calculated nitrogen of a single particle.

The data indicate that a relatively small number of virus particles, of the order of 1 to 100, are capable of initiating a tumor in a susceptible chicken, and that the number required for the 50-percent-infectivity point with the agent of chicken tumor I is considerably less than that required with another tumor-inducing agent, the virus of rabbit papilloma.

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ANNOUNCEMENT

BRITISH AMERICAN EXCHANGE FELLOWSHIPS IN CANCER RESEARCH

Awarded Upon Recommendation of the Committee on Growth of the National Research Council

British American Exchange Fellowships in Cancer Research of the American Cancer Society, awarded by the Society upon recommendation of the Committee on Growth of the National Research Council, are offered to citizens of the United States for advanced training and experience in Great Britain in specialized fields of investigation pertaining to the problem of cancer. Similar fellowships are awarded by the British Empire Cancer Campaign to British scientists for study in the United States. The Society and the Committee on Growth welcome the cooperation of universities and hospitals in making known these opportunities to suitable candidates.

Fields of Study

These fellowships are awarded by the American Cancer Society to provide specialized training for American investigators in Great Britain where opportunities exist for study in facets of research in malignant disease not widely available in this country. Training for an equal number of young British scientists selected by the Campaign will be provided in this country. The Society, the Committee on Growth, and the British Empire Cancer Campaign believe that the training of young men and women in the many complex disciplines of modern scientific thought and techniques are fundamental to a sound approach to the problem of human cancer.

Candidates will be favored who express intention of following a career in a discipline applicable to the study of growth. In evaluating fellowship applications the Committee will keep in mind potential applicability of the proposed training program to problems of neoplastic growth in specialized scientific areas in which superior facilities exist in Great Britain.

Qualifications of Applicants

Fellowships are open to citizens of the United States who possess the degree of Doctor of Medicine, Doctor of Philosophy, or Doctor of Science. They are intended for young men and women embarking upon a career in clinical medicine or basic research, and also for more mature candidates desiring to extend their fields of competence in these fields. Applicants must be able to demonstrate that they have qualifications necessary to pursue investigation in the fundamental sciences or in clinical medicine.

Applications should state the institution where the fellow plans to work in Great Britain; the individual under whom the fellow desires to work; what problem he intends to investigate; when he wishes to start.

Conditions of Appointment

Fellowships will be awarded for a period of one year.

The annual stipend will be £1000 ($4,020.00). An allowance of $600.00 is made for travel to the site of the fellowship in Great Britain.

University staff appointment, with teaching duties agreeable to the fellow, is permitted, provided it carries no additional salary and provided it is acceptable to the Committee on Growth, the American Cancer Society, and the British Empire Cancer Campaign. No other remunerative work will be permitted during the tenure of the fellowship.

Fellowship appointments are subject to the condition that once accepted, they will not be vacated or the place of work changed within the period of tenure without the consent of the Committee on Growth, the American Cancer Society, and the British Empire Cancer Campaign.

Time of Application

Application forms for British American Exchange Fellowships in Cancer Research may be procured from and submitted at any time to the Executive Secretary of the Committee on Growth, Division of Medical Sciences, National Research Council, 2101 Constitution Ave., Washington 25, D. C. Favorable action by the Committee on Growth takes the form of a recommendation to the American Cancer Society, and is subject to approval by the Society. Fellowships will be made effective at the convenience of the institution and the fellow.

Location of Work

British American Exchange Fellowships in Cancer Research are not granted to institutions. The place where the applicant chooses to work and the person under whose guidance he wishes to work must be approved by the Committee on Growth and the British Empire Cancer Campaign. Universities or institutions in which fellows plan to work will supply necessary facilities and equipment.

Reports

Within thirty days after the termination of each year of the fellowship, the fellow is required to submit to the Committee on Growth a report of his work during that period.

Publications

It is understood that results of work carried out by a fellow shall be available to the public through the approved scientific channels without restriction. Publications should include a statement that the investigation was carried out during tenure of a British American Exchange Fellowship in Cancer Research of the American Cancer Society and the British Empire Cancer Campaign, recommended by the Committee on Growth of the National Research Council. The Comptroller, American Cancer Society; Executive Secretary of the Committee on Growth; and the General Secretary of the British Empire Cancer Campaign should be notified immediately of acceptance of manuscripts for publication, in order that reprints may be ordered.

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